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MICROSPECTROPHOTOMETRIC ANALYSIS OF METACHROMATIC STAINING OF NUCLEIC ACIDS[1]

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METACHROMASIA is one of the most striking and useful of the many techniques of staining histological elements. With certain basic dyes, some acid tissue constituents appear colored like the dye in dilute aqueous solution (i.e., stain *orthochromatically*), while others are colored very differently (stain *metachromatically*). With toluidine blue, for example, typical orthochromatic elements are cell nuclei (blue), while a metachromatic (red) color characterizes the matrix of cartilage, mucous secretion of glands, and the granules of mast cells. The term "chromotrope" has been given to those substances which have the capacity for staining metachromatically. The old histological findings have been confirmed by studies of the effect of substances extracted from tissue on the color of dye solutions; Kelley and Miller (1935) compared the thionin staining of smears of purified mucin and thymus desoxyribonucleoprotein and found that the former was metachromatic, the latter orthochromatic. As a result of extensive *in vitro* experiments, Lison (1935) suggested that all chromotropes encountered in histological preparations are half-esters of sulphuric acid with high-molecular-weight carbohydrates. Bank and Bungenberg de Jong (1939) indicated that phosphoric acid ester colloids, such as yeast nucleic acid, also act as chromotropes in solution, although to a lesser extent than the sulphuric acid esters. The metachromatic color was considered to be due to an aggregation of dye molecules, through Van der Waals forces, subsequent to the electrostatic association with the chromotropic substrate. Michaelis and Granick (1945) analyzed the changes in absorption curves characteristic of metachromatic dyes. These dyes in aqueous solution fail to follow Beer's law; that is, new absorption peaks appear as the concentration of the dye is increased, instead of the extinction or optical density at any wave length being strictly proportional to dye content. Physicochemical experiments (Rabinowitch and Epstein, 1941; Sheppard, 1942) suggest that this change is due to reversible dye polymerization, the predominant absorption peak in dilute solu-

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tion (α-peak) being due to the monomeric form of the dye, while, as concentration increases, an absorption peak at shorter wave length due to dimer formation (the β-peak) becomes prominent; while in very high dye concentrations or in the presence of chromotropes higher states of aggregation may cause the appearance of absorption at still shorter wave lengths (the μ-peak). Michaelis and Granick considered that both nucleic acids are model orthochromatic substances by virtue of their phosphoric acid distribution, which prevents dye interaction. A contrary view was expressed recently by Lison and Mutsaars (1950), who reported that *in vitro*, with certain proportions of dye to nucleic acid, either nucleic acid may cause a metachromatic shift of the absorption curve of toluidine blue solutions. (At high nucleic acid concentrations they found a reversal to orthochromasy; the absorption curve of the dye–nucleic acid solution was shifted farther from metachromasy than that of the dilute dye solution alone.)

Whether nucleic acids in tissues act as chromotropes is an equally controversial subject. Many authors (Kelley and Miller, 1935; Holmgren, 1940; Michaelis and Granick, 1945; Sylven, 1950) believe they are not, but Wislocki, Bunting, and Dempsey (1947) have reported the chromotropic activity of ribonucleic acid in certain cell types. The present authors reported briefly (Flax and Himes, 1950; Himes and Flax, 1950) that in a number of plant and animal tissues ribonucleic acid stains metachromatically, desoxyribonucleic acid relatively orthochromatically. An analysis of the metachromatic behavior of nucleic acids under various staining conditions will be reported here. Absorption curves of stained histological preparations have been determined in order to give some insight into the problem of the chromotropic nature of the nucleic acids and the physical basis of metachromasy.

EXPERIMENTAL

METHODS

Azure B bromide (Nat. Anil. Div., Cert. No. NAb2, 88 per cent dye content), a basic thiazine dye, has been used in the following studies on metachromasy. In the earlier work (Flax and Himes, 1950) a metachromatic dye was prepared by chloroform extraction of Azure A (Nat. Anil. Div., Cert. No. NAz14, 92 per cent dye content) and potassium acid phthalate in aqueous solution. This dye was later identified as Azure B, which was either an impurity in the Azure A sample or was formed from it by the method of preparation. Azure B appears to be especially useful for the study of metachromasy, since the orthochromatic and metachromatic absorption peaks are farther apart than those of the commonly used metachromatic dyes, allowing the two colors to be more easily distinguished. However, some other metachromatic dyes show properties entirely similar to those of Azure B.

A large variety of plant and animal tissues were used as test materials. All were fixed in Carnoy's fluid (3 parts absolute ethyl alcohol: 1 part glacial acetic acid) for 1–3 hours, imbedded in paraffin, and sectioned at from 4 to 12 μ. The staining of isolated DNA-histone and RNA–egg albumin fibers was also studied. DNA-histone (prepared from calf thymus by the method of Mirsky and Pollister [1946]), and Schwarz RNA with purified egg albumin were made into fibers by the technique of Devaux (1935): a few drops of each nucleoprotein solution were spread on the surface of a saturated ammonium sulphate solution and compressed into an insoluble fiber. These fibers were then treated in the same manner as the fresh tissues.

Slides were stained (from 1 to 24 hours) in various concentrations of dye made up in citrate or potassium acid phthalate buffers. They were then washed briefly in water, differentiated overnight in tertiary butyl alcohol (in order to assure only chemical staining, according to Michaelis [1947]), transferred to xylol, and mounted in clarite.

In order to analyze the specificity of staining, ribonuclease and desoxyribonuclease, obtained from Worthington Biochemical Laboratory, were used. The RNase was boiled for 5 minutes in a few drops of saturated ammonium sulphate to remove proteolytic impurities (Kaufmann *et al.*, 1947) and diluted in water to a concentration of 0.2 mg/cc. DNase, 0.1 mg/cc in phosphate buffer at pH 7.5, was prepared with gelatin (0.1 per cent) and 0.03 M magnesium sulphate (McCarty, 1946). Slides were digested with ribonuclease for 4 hours at 45° C. prior to staining for complete RNA removal, while the conditions for complete DNA digestion were 3 hours at room temperature. In all cases controls for the enzyme experiments were carried out simultaneously; these slides were treated under the same conditions except for the absence of the enzyme.

The absorption spectra of Azure B in solution were analyzed by means of a Beckman spectrophotometer, using cuvettes of 1- and 0.02-cm. thickness. In tissues, absorption curves of bound Azure B were obtained by measurements with a photometric apparatus similar to that described by Pollister (1952) (see Fig. 1). The source used was a G.E. pro-

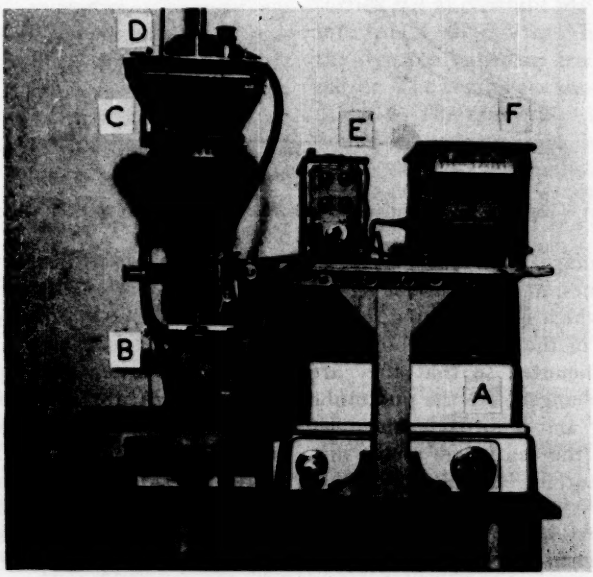

FIG. 1.—Apparatus for absorption measurements of cells. The major components are as follows: *A*, Perkin-Elmer model monochromator, with crystal quartz prism; *B*, Bausch and Lomb Model DDE microscope, with specially fitted tube for lateral observation; *C*, Zeiss photomicrographic camera; *D*, Farrand photometer with IP21 photomultiplier; *E*, battery and control unit; and *F*, Rubicon galvanometer.

jection lamp (code 18AT10P-6V) regulated by a Raytheon voltage stabilizer. The combination of an achromatic lens close to the exit slit and an apochromatic objective used as a microscope condenser forms an image of the exit slit in the plane of the cell on a glass microscopic slide. The microscope, with a 90× apochromatic objective and 25× eyepiece, projects the image of cell and slit, magnified 2,500 times, at the plane of an iris diaphragm, which is substituted for the ground glass of the camera. Above this diaphragm, mounted so that they are readily interchanged, are the phototube compartment and a focusing magnifier for centering the cell image in the diaphragm aperture.

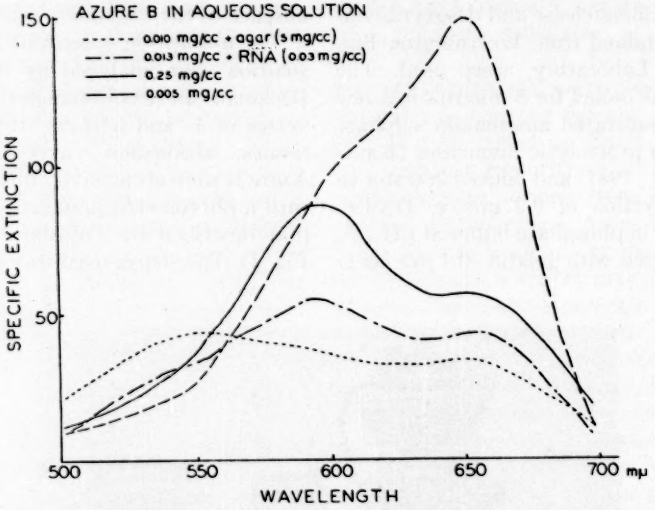

FIG. 2.—Effect of changing concentration and substrate on absorption curves of Azure B in solution

RESULTS

I. METACHROMATIC PROPERTIES OF AZURE B IN SOLUTION

The absorption spectra of Azure B in aqueous solution are similar to those of other metachromatic dyes (Michaelis and Granick, 1945) in that this dye does not follow Beer's law, as shown in Figure 2. The ordinate is expressed as the optical density, or extinction, of 1 mg/cc of dye in a 1-cm. light path (specific extinction). A cuvette 1 cm. thick was used for dye concentrations of 0.013 mg/cc or less, a 0.2-cm. cuvette for 0.25 mg/cc dye concentration. The orthochromatic peak at 650 mμ is evident in dilute solution. Instead of the absorption at this peak increasing proportionally with increasing concentration, so that the specific extinction remains constant (i.e., obeying Beer's law), a new peak appears at 590 mμ. When Azure B is combined with agar, or with RNA, using concentrations similar to those used by Di Marco and Boretti (1950), a third peak appears in the region of 540 mμ. It can be seen that the specific extinction drops markedly as the highest absorption peak is shifted toward lower wave lengths. In alcoholic solution, this change in specific extinction and in shape of absorption curves does not occur.

Another property of metachromatic

dye solutions is the loss of metachromatic absorption as a result of increasing temperature. If a solution of Azure B and agar (showing the metachromatic color) is heated, there is a loss of metachromatic absorption, so that the color becomes identical with that of the orthochromatic, dilute dye solution. This process is completely reversible; the metachromatic color returns on cooling.

When the pH of an Azure B solution is made highly alkaline (above pH 9), the absorption is shifted greatly in the metachromatic direction. This change is due presumably to free base formation, which is considered unrelated to "true metachromasy," since the absorption curve is not similar to that of the concentrated dye solution or that of the dye combined with agar (Michaelis and Granick, 1945).

II. METACHROMATIC BEHAVIOR OF AZURE B IN TISSUES

A. SPECIFICITY OF STAINING

Azure B combines with nucleic acids and certain acid polysaccharides when the staining is carried out at pH 4 or lower, and also with acid proteins at higher pH. Protein staining is negligible at pH 4, as demonstrated by the absence of all staining except that of localized polysaccharide esters (i.e., cartilage matrix, mast cells) following the specific removal of nucleic acids. The methods used to remove both ribose and desoxyribose nucleic acids are (1) treatment with boiling 5 per cent trichloracetic acid for 15 minutes (Schneider, 1945; Pollister and Ris, 1947) and (2) digestion with ribonuclease and desoxyribonuclease prior to staining. Thus, since polysaccharide staining can be readily recognized, Azure B may be used at pH 4 or lower as a specific stain for nucleic acids.

B. EFFECTS OF VARYING THE DYE SOLUTION

Azure B behaves like other metachromatic dyes in staining sulphuric acid esters metachromatically. In addition, under specific conditions, nucleic acids may also stain relatively metachromatically. This is not prevented by alcohol differentiation. The effect of varying the concentration, pH, and temperature of the staining solution was investigated to determine the manner in which these conditions alter the metachromatic staining of nucleic acids. In order to study the staining of ribose and desoxyribose nucleic acid separately, cytoplasm and nucleoli of corn microsporocytes and RNA fibers were used for the former study, while trout sperm heads (which chemical analysis has shown to contain no RNA) and DNA fibers were used for the latter.

1. *The effect of dye concentration.*—The concentration of Azure B has a considerable effect on the shape of the absorption curve of the dye combined with a particular tissue component. Staining was carried out at constant pH and temperature (pH 4, 40° C.) for 3 hours, varying the dye concentration from 0.05 to 2.0 mg/cc. The results are shown in Figures 3 and 4. The absorption curves of dye bound to tissue nucleic acids show peaks at the same wave lengths as the dye in solution: the α-peak at 650 mμ, the β-peak at 590 mμ, and the third peak at about 545 mμ. In addition to each complete curve, at least ten measurements at the three peak wave lengths were made. These indicated that the complete curves were characteristic.

At a very low dye concentration (0.05 mg/cc), the stained RNA and DNA both appear blue-green; however, while the absorption curve of the DNA-dye complex is similar to the orthochromatic curve of the dilute dye solution (Fig. 2), that of the dye bound to RNA appears more metachromatic, with relatively higher absorption at 590 mμ. At high dye concentrations (2.0 mg/cc) both RNA and DNA stain a metachromatic, purple color, showing considerable absorption at

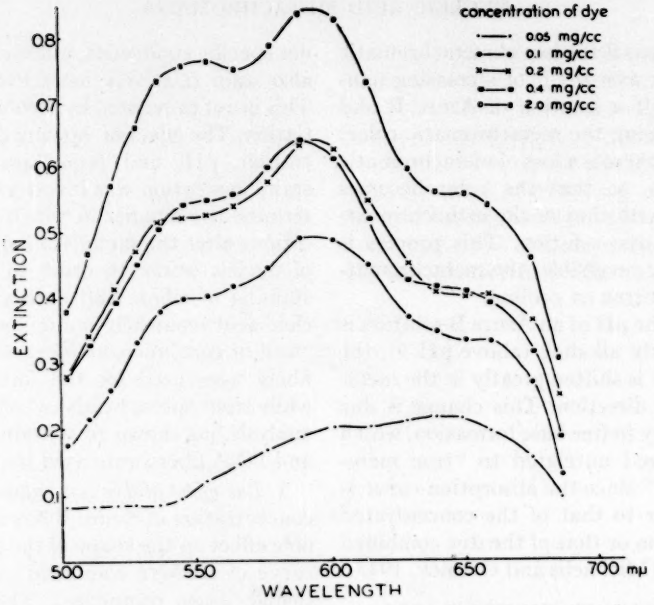

FIG. 3.—Effect of concentration of Azure B on RNA metachromasy. Corn-bud sections (8 μ) stained with various concentrations of Azure B at pH 4; absorption measurements through microsporocyte cytoplasm.

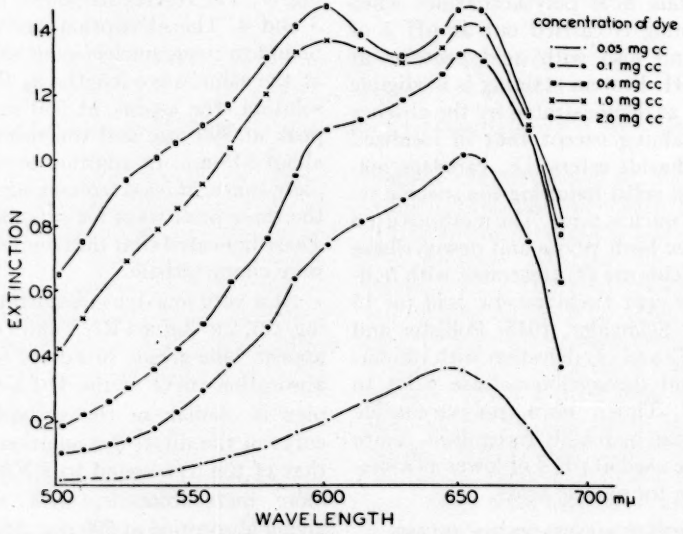

FIG. 4.—Effect of concentration of Azure B on DNA metachromasy. Trout testis sections (9 μ) stained as in Fig. 3; absorption measurements through mature sperm heads.

590 and 545 mμ, but RNA appears more metachromatic than DNA. The transition between these two types of curves occurs at intermediate concentrations of staining solution; the two nucleic acids always act in clearly different manners at each concentration. RNA induces a metachromatic color when stained with 0.2 mg/cc dye, while, with DNA, no clear metachromatic staining occurs until a concentration of dye of 1.0 mg/cc is used.

2. *The effect of pH of staining medium.* —Staining was carried out in Azure B (0.25 mg/cc) of various acidities for 3 hours at 40° C. Within the pH range used (pH 3–6) there is no change in absorption properties of the dye solution. Absorption curves of the dye combined with RNA and DNA were determined. The date are presented in Tables 1 and 2 in the form of ratios of extinction at three wave lengths near the three absorption peaks. Each ratio represents the mean of at least ten measurements at the two wave lengths compared. The mean extinction at one wave length of the curve is also presented in order to indicate the changes in amount of dye bound.

Increasing the pH of the staining solution increases the amount of metachromasy, as evidenced by the higher ratios, indicating a higher proportion of 546 mμ absorption. This effect is similar to that of high dye concentration, in that both cause an increase in the amount of dye bound to the tissue which is accompanied by a higher degree of metachromasy. Both nucleic acids stain essentially orthochromatically at pH 3 or lower, while both are metachromatic above pH 5. Above pH 4, acid groups of proteins stain with a purely orthochromatic absorption, which may account for the lowering of the RNA–Azure B ratios given in Table 1. This protein staining, however, does not obscure the change in DNA staining to a metachromatic, purple color. The shift toward greater metachromasy occurs at different pH ranges; the shift in RNA occurs between pH 3 and pH 4, while that of DNA occurs between pH 5 and pH 6.

Ribonucleic acids of different sources show some variation in the pH range at which the shift to inducing metachroma-

TABLE 1

EFFECT OF pH OF STAINING SOLUTION ON RNA METACHROMASY*

pH	MEAN E_{546}/E_{590}		MEAN E_{546}/E_{453}		MEAN E_{590}	
	Nucleolus	Cytoplasm	Nucleolus	Cytoplasm	Nucleolus	Cytoplasm
3.05.....	0.37	0.41	0.24	0.34	0.139	0.056
3.5.....	0.40	0.73	0.34	1.04	0.223	0.339
3.8.....	0.79	0.87	1.31	1.47	0.759	0.470
4.0.....	0.83	0.87	1.46	1.53	1.15	0.510
4.2.....	0.79	0.84	1.29	1.40	1.21	0.716

* Corn microsporocytes (8 μ) stained in Azure B, 0.2 mg/cc, 40° C., 2 hours.

TABLE 2

EFFECT OF pH OF STAINING SOLUTION ON DNA METACHROMASY*

pH	Mean E_{546}/E_{590}	Mean E_{546}/E_{453}	Mean E_{453}
4.0.....	0.33	0.21	0.770
5.1.....	0.80	0.90	0.906
5.8.....	0.92	1.18	1.04

* DNA-histone fibers (6 μ) stained in Azure B, 0.2 mg/cc, 40° C., 2 hours.

sy occurs. Comparison of the two types of RNA analyzed in Table 1 indicates that the nucleolar RNA becomes metachromatic at a pH higher than that of the cytoplasm, while other ribonucleoproteins analyzed, such as yeast RNA or mouse Nissl substance, show slight variations within this range, suggesting that this effect may be of value in characterizing different ribonucleoproteins.

The possibility that the decrease in metachromasy and in amount of dye bound might be due to a loss of nucleic acid phosphorus during staining was eliminated in the following way: a slide was stained at pH 3, then restained at pH 4; the amount of dye combined with the nucleic acid was the same as that of an untreated slide stained at pH 4.

3. *The effect of the temperature of the staining solution.*—The tissues used previously for studying RNA metachromasy were stained at various temperatures, from 7° to 90° C. in 0.4 mg/cc Azure B for 1 hour. Complete absorption curves of stained corn microsporocyte cytoplasm are given in Figure 5. The results indicate that increasing the temperature of the dye solution up to about 60° C. tends to increase both the amount of dye combined and the amount of metachromasy, although only a small change in metachromasy occurs in this range. However, at a very high staining temperature (90° C.) the amount of staining is decreased and the RNA appears an orthochromatic, blue-green color. The possibility that the decrease in staining and metachromasy might be due to loss of RNA from the tissues was eliminated by an experiment analogous to that performed to test the effect of low pH. After staining a slide at 90° C. (resulting in orthochromatic RNA staining), it was restained at 40° C. An intense, metachromatic staining of RNA was observed which was identical with that of a control slide stained only at 40° C. Therefore, the orthochromatic staining at 90° C. cannot be due to a loss of RNA.

C. AZURE B AS A DIFFERENTIAL STAIN FOR NUCLEIC ACIDS

Varying the concentration, pH, and temperature of the staining solution has shown that under some conditions the chromotropic activity of RNA and DNA is affected to different extents. This gives rise to the difference in color which is easily detected by eye. The ideal conditions for the use of Azure B as a differential stain for the two nucleic acids are: 0.25 mg/cc concentration, pH 4.0, for 2 hours at 40° C. Under these conditions RNA stains purple, while DNA stains greenish blue.

Three types of evidence of the specificity of this differential stain were obtained: (1) The distribution of colors within the cell is identical with the known distribution of DNA and RNA in cells; the nuclei appear blue-green and the cytoplasm and nucleoli are purple. (2) The use of ribonuclease and desoxyribonuclease independently established that all the purple staining was absent following RNase digestion, while all blue-green staining was prevented by DNase. (3) The absorption curves of chemically defined substrates (fibers of RNA-albumin and DNA-histone) stained with Azure B appear identical to those of suspected RNA and DNA in tissues, as shown in Figure 6. Furthermore, the absorption curves of Azure B combined with different metachromatic substrates (cartilage or mucin) are clearly different from RNA (Fig. 7).

D. EFFECT OF SUBSTRATE ON METACHROMASY

It is clear from *in vitro* work with metachromatic dyes (Kelley and Miller, 1935; Michaelis and Granick, 1945; Lison and Mutsaars, 1950) that changing the concentration of the chromotrope

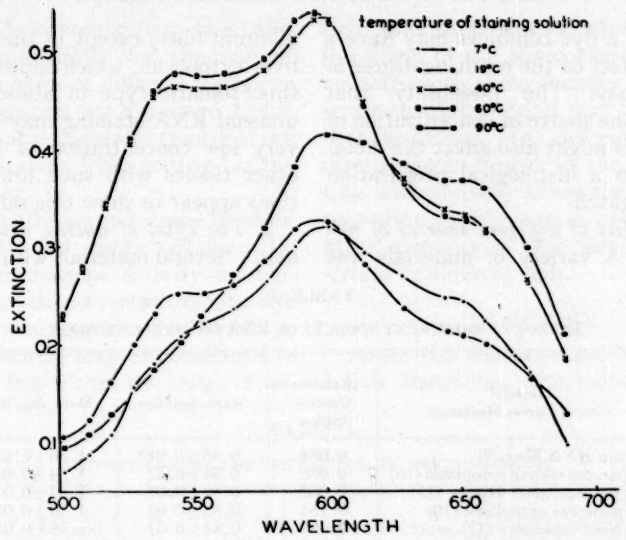

FIG. 5.—Effects of temperature of staining solution on RNA metachromasy. Corn buds (8 μ) stained in Azure B, 0.4 mg/cc, pH 4, for 1 hour at various temperatures. Absorption curves measured through microsporocyte cytoplasm.

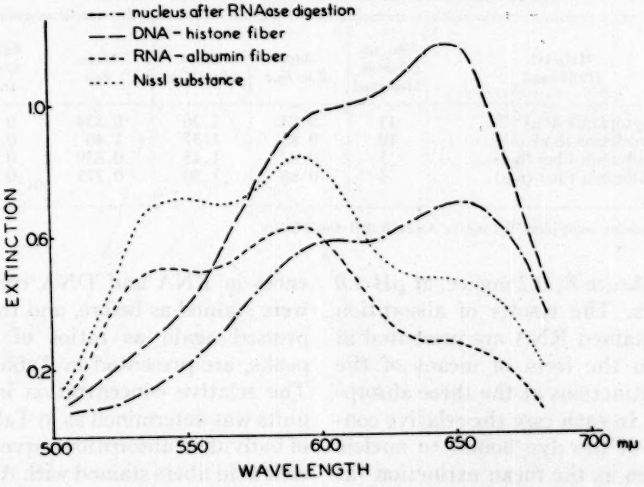

FIG. 6.—Differential metachromasy of RNA and DNA bound to Azure B. Ambystoma tissues (8 μ) compared with artificial RNA and DNA fibers (6 μ) stained with Azure B at pH 4, 0.2 mg/cc concentrations, for 2 hours at 40° C.

with which a dye combines may have a profound effect on the resulting degree of metachromasy. The possibility that changes in the source or concentration of nucleic acids might also affect the metachromasy in a histological preparation was investigated.

1. *The effect of different sources of nucleic acid.*—A variety of materials was stained in Azure B, 0.2 mg/cc, at pH 4.0 for 2 hours. The results of absorption curves of stained RNA are presented in Table 3, in the form of means of the ratios of extinctions at the three absorption peaks. In each case the relative concentration of the dye bound to nucleic acid is given as the mean extinction (at the wave length of the highest absorption) divided by the section thickness. The statistical evaluation of the ratios of extinctions shows no significant deviation from that of the standard RNA–egg albumin fiber, except in the case of the liver cytoplasm, which shows a more orthochromatic type of absorption. This unusual RNA staining may be due to a very low concentration of RNA, since other tissues with such low concentrations appear to show this same effect.

2. *The effect of nucleic acid concentration.*—Several materials with wide differences in RNA and DNA concentration were stained as before, and the data, expressed again as ratios of absorption peaks, are presented in Tables 4 and 5. The relative concentration in arbitrary units was determined as in Table 3. Data of individual absorption curves of the nucleic acid fibers stained with Azure B also show a great range in concentration. It is evident that within these ranges of substrate concentration there are no significant differences in the shape of the absorption curves.

TABLE 3

EFFECT OF DIFFERENT SOURCES OF RNA ON METACHROMASY

Material (No. of Curves Measured)	Relative Concentration	Mean E_{544}/E_{590}	Mean E_{544}/E_{493}
Schwarz RNA fiber (9)	0.084	0.86±0.01*	1.56±0.05
Ambystoma oöcyte cytoplasm (10)	0.086	0.84±0.02	1.34±0.04
Corn nucleus, after DNase (15)	0.083	0.86±0.02	1.63±0.07
Frog pancreas cytoplasm (10)	0.154	0.82±0.02	1.40±0.04
Frog Nissl substance (12)	0.091	0.82±0.02	1.35±0.06
Frog liver cytoplasm (10)	0.017	0.68±0.04	0.97±0.10

* Means are presented with their standard errors.

TABLE 4

EFFECT OF RNA CONCENTRATION ON METACHROMASY*

Material (Thickness)	No. of Curves Measured	Mean E_{544}/E_{590}	Mean E_{544}/E_{632}	Mean E_{590}	Relative Concentration
Corn cytoplasm (8 μ)	11	0.84	1.26	0.534	0.067
Corn nucleolus (6 μ)	10	0.85	1.37	1.40	0.233
RNA-albumin fiber (6 μ)	1	0.82	1.45	0.839	0.140
RNA-albumin fiber (6 μ)	1	0.86	1.50	0.273	0.045

* Staining conditions: 0.4 mg/cc Azure B; pH 4.0; 2 hours.

The DNA concentration was also varied experimentally by the use of DNase. The effect of different durations of DNase digestion was examined, and it was found that no apparent change in the color of the stained DNA occurred as the DNA was lost.

There is a striking difference between these results and those obtained by studying chromotrope activity in solution. In fact, unlike a metachromatic dye in aqueous solution, the dye bound to tissue nucleic acid may be considered to obey Beer's law within the range of nucleic acid concentration studied. Since the shape of the absorption curve does not vary with these changes of the substrate concentration, it may be assumed that the extinction at any wave length is directly proportional to the concentration. This property allows the use of photometric measurements to determine relative amounts of dye bound to substrates in different cells.

DISCUSSION

Azure B is characterized by failure to follow Beer's law in aqueous solution.

TABLE 5

EFFECT OF DNA CONCENTRATION ON METACHROMASY*

Material (Thickness)	No. of Curves Measured	Mean E_{544}/E_{590}	Mean E_{544}/E_{632}	Mean E_{632}	Relative Concentration
Trout sperm nuclei (9 μ)	9	0.73	0.71	1.13	0.126
Corn nuclei, after RNase (8 μ)	10	0.76	0.77	0.289	0.036
DNA-histone fiber (6 μ)	1	0.68	0.73	1.48	0.247
DNA-histone fiber (6 μ)	1	0.71	0.75	0.514	0.086

* Staining conditions: 0.4 mg/cc Azure B; pH 4.0; 2 hours.

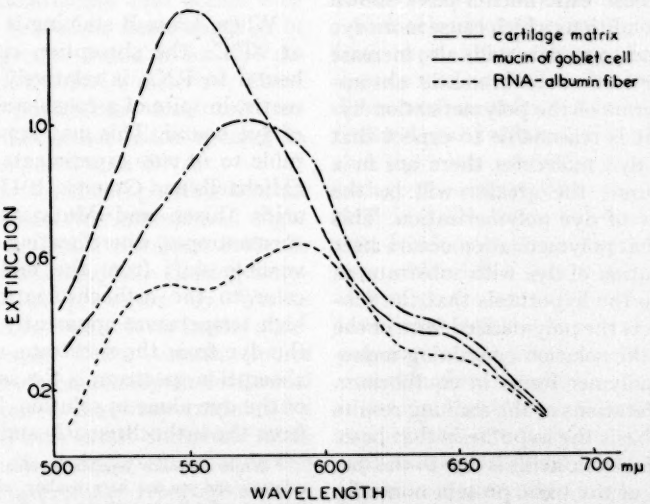

FIG. 7.—Absorption spectra of Azure B combined with sulphuric acid esters of polysaccharides compared to RNA, stained under the same conditions.

New absorption peaks appear as the concentration of the dye is increased, instead of the extinction at any wave length increasing proportionally. In aqueous solution it shows an α-peak at 650 mμ, a β-peak at 590 mμ, and a μ-absorption peak at 545 mμ when the dye is combined with a chromotrope.

The absorption curves of Azure B bound to nucleic acid in tissues all show the 650 mμ α-peak and, in general, the 590 mμ β-peak. Under certain conditions, in addition to the α- and β-peaks, the third μ-peak characteristically appears at 545 mμ. As the extent of metachromasy changes, there is a change in relative heights of these three peaks, rather than a gradual shifting of absorption peaks. The similarity of the absorption peaks of the dye bound to tissue with those of the dye in solution strongly suggests that they also result from dye polymerization.

Further evidence in support of the dye polymerization theory of metachromasy comes from analyzing the effect of changes in the staining conditions. In general, these experiments have shown that the conditions which cause more dye to be bound to nucleic acids also increase the amount of metachromatic absorption. In terms of the polymerization hypothesis, it is reasonable to expect that the more dye molecules there are in a given volume, the greater will be the probability of dye polymerization. This assumes that polymerization occurs after the association of dye with substrate, in contrast to the hypothesis that the substrate selects the polymerized form of the dye from the solution containing monomer and polymer forms in equilibrium. All interpretations of the staining results have as a basis the hypothesis that basic staining of nucleic acids is due to the displacement of the basic protein normally associated with the nucleic acid by the basic dye (Michaelis, 1947); the amount of staining depends on the equilibrium[3] reached after competition between these two basic substances for the acid groups of nucleic acid.

It appears that increasing concentration of the dye, pH, and temperature (up to 60° C.) increases metachromasy by increasing the number of phosphate groups of nucleic acid available for dye combination, which allows greater dye interaction subsequent to combination with the substrate. This concept of metachromasy was clearly expressed by Bank and Bungenberg de Jong (1939), who considered the "charge density" of the substrate the determining factor for metachromasy. In the case of nucleic acids, the "charge density" is equivalent to the number of available phosphate groups per unit volume capable of combining with the basic dye.

There are two cases which indicate that the total amount of dye bound is not the sole determining factor for metachromasy.

THE TEMPERATURE EFFECT

When Azure B staining is carried out at 90° C., the absorption curve of dye bound to RNA is relatively orthochromatic, in spite of a considerable amount of dye bound. This may appear comparable to *in vitro* experiments using agar (Michaelis and Granick, 1945) or nucleic acids (Lison and Mutsaars, 1950) as chromotropes, where heating causes a reversible shift from the metachromatic color to the orthochromatic one. The high temperature apparently dissociates the dye from the substrate, so that the absorption spectrum is the same as that of the dye alone in solution. This differs from the orthochromatic staining in tis-

[3] While mass-law equilibria refer to systems in solution and we are here dealing with a reaction between solid tissue sections and dye in solution, it seems useful to apply the classical ideas to this more complex system.

sues where the dye remains bound to nucleic acid while the dye aggregates appear to be dissociated. It seems similar, rather, to the phenomenon described by Sheppard (1942), who found that heating a concentrated aqueous solution of the dye shifts the equilibrium from metachromasy to orthochromasy, by preventing dimer formation. In tissues, while the high temperature does not completely prevent the dye-substrate electrostatic bonding, dye polymerization is inhibited. This supports the idea that Van der Waals forces may be involved in the polymerization, since these would be more easily disrupted by heat than would the electrostatic forces.

THE DIFFERENCE BETWEEN RNA AND DNA IN FIXED TISSUES

There is a striking difference between the chromotropic behavior of RNA and of DNA when they are stained with Azure B under the same conditions. This difference does not appear to be solely dependent on the amount of dye bound. The greater metachromasy that occurs with RNA may be due to closer spacings of chosphate groups available for dye attachment in the RNA molecule. There pre several possibilities that might acaount for this closer anion spacing in RNA as compared with DNA.

a) It was considered possible that an excess phosphate (metaphosphate) might be present in RNA. Excellent evidence for such a metaphosphate causing metachromasy was presented by Wiame (1947), who studied volutin bodies of recharged yeast. These volutin bodies stained metachromatically, and chemical analysis showed that they contained a nucleic acid with excess phosphate labile to mild acid hydrolysis. However, in the case of metachromasy reported here, it appears that a similar metaphosphate cannot account for the staining, since it is unaffected by short N/10 HCl hydrolysis and since RNA prepared by Schwarz also shows characteristic metachromatic staining without containing metaphosphate.

b) The difference in degree of polymerization of the nucleic acids might account for the difference in metachromasy, since terminal phosphate groups provide extra sites for basic dye attachment, because of additional (secondary) acid dissociations. It is believed that RNA is a lower polymer than DNA and would therefore contain a greater proportion of terminal phosphate groups. This hypothesis was tested by investigating the effect of DNase on the metachromasy of DNA. The enzyme, in depolymerizing the nucleic acid, should form more terminal groups which would result in greater metachromasy. No increase in metachromasy of DNA occurred following enzyme digestion. Nevertheless, it is possible that the depolymerized fragments may diffuse away immediately and therefore not cause an increase in terminal groups and in metachromasy.

c) A third hypothesis concerns the differences in intramolecular structure of the two nucleic acids as the basis for the differences in inducing metachromasy. Gulland and Jordan (1947) have shown that there is a greater proportion of secondary phosphoryl dissociations in RNA than in DNA, from which they deduce that RNA must have a branched structure while DNA has a straight chain molecular structure. This structural difference (as well as a possible difference in polymerization) would account for extra sites for dye association in the RNA molecule. However, some evidence indicates that a dye molecule can associate with either a primary or a secondary phosphoryl group of a single nucleotide, but not with both (Cavalieri *et al.*, 1951). Nevertheless, the differential ability of

RNA and DNA to induce metachromasy is pH-dependent.

Irrespective of the mechanism, the differential chromotropic activity appears to result from differences in the density of available phosphate groups.

The difference between investigating the chromotropic activity of substrates combined with metachromatic dyes in tissue sections and in solution must be evaluated. *In vitro* experiments have shown that varying the concentration of the chromotrope, while keeping the dye concentration constant, affects the absorption curve to a great extent. By contrast, the data presented in Tables 4 and 5 show clearly that, in histological preparations, changes in nucleic acid concentration have no considerable effect on the absorption curves of the dye–nucleic acid complex.[4] The reason for this apparent discrepancy may be that excess dye is removed in staining, while in solution the absorption spectrum is the sum of that of the dye combined with the chromotrope (a metachromatic curve) and that of the unbound dye (an orthochromatic curve). Therefore, by changing the concentration of the chromotrope in solution, the proportion of bound dye will be altered. Increasing the concentration of substrate shifts the equilibrium to more total dye bound and therefore increases metachromasy. An optimum concentration may be reached so that further increase in substrate concentration will shift the absorption curve to orthochromasy (Lison and Mutsaars, 1950). In this case, so many sites may be available for binding dye that, with random combination, the bound dye molecules are scattered, thereby preventing interaction and polymerization. In the work presented here, the tissue absorption curves are not contaminated by the absorption of uncombined dye, since all excess dye is removed in the process of alcohol differentiation. In the range examined, increasing the chromotrope concentration increases the amount of dye bound, but the absorption curve is not changed. This discrepancy between histological and *in vitro* work with metachromatic dyes may be resolved by performing *in vitro* experiments more closely resembling the staining experiments, where uncombined dye is determined. Such an experiment has been undertaken (Cavalieri and Angelos, 1950); however, a nonmetachromatic basic dye was used.

[4] Lison and Mutsaars (1950) report that the concentration of nucleic acids in tissues does affect the amount of metachromasy. For example, they conclude that the orthochromatic staining of sperm heads is due to the high nucleic acid concentration in proportion to the dye concentration. We also find that trout sperm heads stain orthochromatically, but we believe this to be due to the absence of RNA, since, after RNase, other, less dense nuclei also stain orthochromatically.

SUMMARY

Azure B, a metachromatic dye, when used at pH 4, stains nucleic acids and acid polysaccharides specifically in Carnoy-fixed plant and animal tissues.

Absorption curves of the dye bound to nucleic acids in tissues were determined by means of a microspectrophotometer. The orthochromatic absorption peak (α-peak) at 650 mμ is always present, while a second, β-peak at 590 mμ and a third, μ-peak near 545 mμ may also occur, the latter giving rise to the metachromatic color observed under some staining conditions. The α- and β-peaks are identical with those of a concentrated aqueous solution of the dye, while the μ-peak is in approximately the same position as the μ-peak of the dye combined to certain chromotropes in solution. The repeated occurrence of these three peaks supports an extension of the theory of metachromasy based on dye polymerization in solution to explain metachromasy in tissues.

The effect of alterations of the staining

conditions appears to be satisfactorily interpreted by this theory. Increasing the concentration of dye, the pH, and the temperature (up to 60° C.) of the staining solution increases metachromasy of nucleic acid. This may result from the increase in the amount of dye bound, which presumably would increase the probability of dye polymerization.

Within the range of dye concentrations of 0.2–0.4 mg/cc at 40° C., RNA stains metachromatically (purple), while DNA stains relatively orthochromatically (blue-green), making this a useful technique for the differential localization of nucleic acids in tissue sections. The implications of this difference in the degree of metachromasy of RNA and DNA in terms of their molecular structure is discussed.

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THE USE OF ELECTROMIGRATION TECHNIQUES IN WASHING AND CONCENTRATING CULTURES OF PARAMECIUM AURELIA[1]

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THE necessity for obtaining concentrated suspensions of paramecia relatively free of bacteria and culture medium has led to the development of electromigration techniques (Packchanian, 1943; Humphrey and Humphrey, 1948; Lubinsky, 1949; Van Wagtendonk and Hackett, 1949) for this purpose. These techniques are based on the observation of Ludloff (1895) that paramecia orient themselves in a direct current and migrate from the anode to the cathode.

EXPERIMENTS AND RESULTS

A number of variations in the apparatus may be employed, but all have the same basic design. The paramecia are migrated from the anode out of a large container, through a narrow tube, into a receiver which is in contact with the cathode. A 110–20-volt direct-current source is used. The electrodes are of the conventional nonpolarizing type, consisting of copper wire in contact with a 10 per cent solution of copper sulphate. This solution is connected to a 10 per cent solution of potassium chloride via an agar bridge consisting of 3 per cent agar and 3 per cent KCl. Finally, contact with the migrating chambers is made via another agar bridge of the same composition.

The principal requirement of the migrating medium is that it should be nontoxic to the paramecia while permitting the migration to proceed. The following migrating solutions have been used: (1) one part of Ringer's solution to 10 parts of exhausted culture fluid (obtained from starved cultures of paramecia by filtration through a Berkefeld filter); (2) one-tenth strength Ringer's solution; and (3) an inorganic salt solution of the following composition: NaCl, 0.04 per cent; KCl, 0.01 per cent; $CaCl_2 \cdot H_2O$, 0.02 per cent; $MgSO_4 \cdot 7H_2O$, 0.01 per cent; K_2HPO_4, 0.005 per cent; and KH_2PO_4, 0.005 per cent. The pH is adjusted to 7.0. This medium is referred to hereafter as "medium 3."

A brief study of the conditions affecting the migration of the paramecia was made. All migrations were run at room temperature ($26° \pm 1°$). *Paramecium aurelia*, variety 4, stock 51, mating type VII (S) was used as the test organism. A variation of the apparatus shown in Figure 1 was used. The agar bridge from the anode makes contact with the culture to be migrated via a standard taper joint (14/35) sealed to the bottom of a test tube, *A* (18 mm. inside diameter). A side arm, *B*, of 6-mm. glass tubing is sealed on

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the opposite side of the test tube, 10 cm. from the bottom. For these tests, a long straight side tube (120 cm.) extending horizontally to the cathode was used. Contact is made with the cathode via another standard taper joint. A stopcock, *C*, is sealed to the side tube near the connection with the cathode. The side tube the individual animals by means of a stop watch. Rates were recorded at 3 and 100 cm. from the point of attachment of the side arm. The rates of swimming at the beginning of the migration path did not vary with the time of exposure to the electric current. However, at the 100-cm. point, the animals migrated

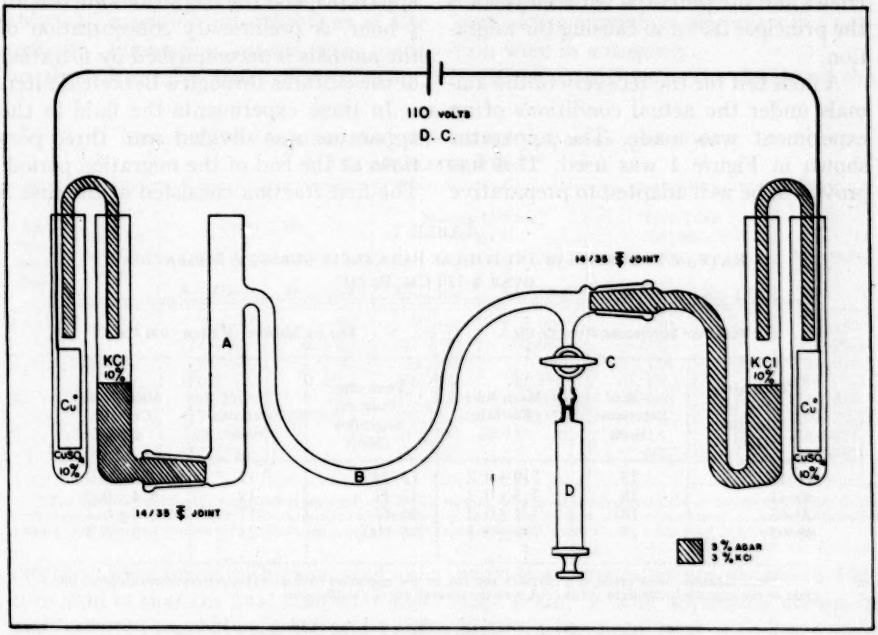

FIG. 1.—The basic migration apparatus

is completely filled with migrating medium by means of a syringe, *D*, attached to the side arm. Twenty-five ml. of the culture to be migrated are then placed in the test tube. Additional migrating medium to make contact with the culture is forced into the apparatus from the cathode end by means of the syringe.

The effect upon the rate of migration of the time of exposure to the electric current and the length of the migration path was determined (Table 1). The time required for the paramecia to migrate across a 0.5-cm. path was measured for more slowly after longer exposures to the current.

The current passing through the apparatus may be controlled by varying the salt concentration. At different current strengths and salt concentrations (Table 2) the rate of swimming of the individual animals does not vary appreciably. The determinations were made at the start of the migration path during two 5-minute intervals after closing the circuit. The rate of swimming was lowered slightly at the highest salt concentration and current strength, but no other effect was

noted. When distilled water was placed in the migration tube, the rate of swimming slightly increased, but the appearance of the animals indicated that the water was causing them to swell. With distilled water, no reading could be obtained on the milliammeter. This indicates that the polarity of the two electrodes and the potential between them is the principal factor in causing the migration.

A final test for the recovery of the animals under the actual conditions of an experiment was made. The apparatus shown in Figure 1 was used. This has proved to be well adapted to preparative procedures and has the advantage that several of these cells can be set up in series between one set of electrodes by means of agar bridges leading from the cathode end of one cell to the anode end of the other, thus making it possible to run several migrations simultaneously. Salt medium 3 is used routinely in this apparatus, and the migrations are run for ½ hour. A preliminary concentration of the animals is accomplished by filtration of the cultures through a Berkefeld filter.

In these experiments the fluid in the apparatus was divided into three portions at the end of the migration period. The first fraction consisted of the first 5

TABLE 1

RATE OF SWIMMING OF INDIVIDUAL PARAMECIA DURING A MIGRATION OVER A 120-CM. PATH*

START OF MIGRATION PATH (3 CM.)			END OF MIGRATION PATH (100 CM.)		
Time after Start of Migration (Min.)	No. of Determinations	Mean Rate (Cm/Min) ±S.E.	Time after Start of Migration (Min.)	No. of Determinations	Mean Rate (Cm/Min) ±S.E.
11–16	13	7.9±0.2	17–22	17	7.3±0.3
36–41	18	7.5±0.2	42–47	19	6.4±0.2
60–65	18	7.1±0.2	66–67	17	5.3±0.4
88–93	17	7.9±0.3	95–100	23	5.9±0.3

* Determinations were made at 3 cm. and 100 cm. in the migration path at various time intervals after the start of the migration. Medium 3 was used, and the current was 0.3 milliampere.

TABLE 2

RATE OF SWIMMING OF INDIVIDUAL PARAMECIA AT DIFFERENT SALT CONCENTRATIONS AND CURRENT STRENGTHS*

RELATIVE SALT CONCENTRATION	CURRENT (MILLIAMPERES)	FIRST 5 MINUTES		SECOND 5 MINUTES	
		No. of Determinations	Rate of Swimming (Cm/Min) ±S.E.	No. of Determinations	Rate of Swimming (Cm/Min) ±S.E.
10×	2.8	14	6.1±0.3	13	7.7±0.3
2×	0.9	13	8.6±0.3	13	9.6±0.4
1×	0.5	10	8.1±0.3	14	8.2±0.4
0.5×	0.3	10	9.4±0.4	12	8.9±0.2
0.33×	0.2	15	8.7±0.4	15	10.8±0.04
Distilled water	0.0	10	10.2±0.1		

* The salt concentrations are expressed as multiples of the concentration of salt in medium 3. Determinations were made at the 3-cm. point in a 60-cm. migration path.

ml. withdrawn from the cathode end of the apparatus. The second fraction (7 ml.) was withdrawn from the U-tube, *B*. The last fraction (30 ml.) was that remaining in the test tube, *A*, and contained the animals which had not migrated. No attempt was made to wash the final residue of paramecia out of the apparatus, and some of the animals probably had migrated into the agar of the cathode. Population counts were made on these three fractions. For counting, dilutions were made with exhausted culture fluid so that the final number of animals counted was between 500 and 1,000 in 1 ml. Four or five counts were made for each determination. No significant difference in counts was noted when the animals were fixed with aceto-carmine stain prior to the dilution.

TABLE 3

RECOVERY OF PARAMECIA FOLLOWING ROUTINE ½-HOUR MIGRATION IN APPARATUS (FIG. 1)*

Experiment No.	Total No. Animals Used	First 5 Ml.		Middle U-Tube (7 Ml.)		Test Tube (30 Ml.)		Recovery
		No. Alive	No. Dead	No. Alive	No. Dead	No. Alive	No. Dead	
1....	6,450	1,433 (22.2%)	30 (0.5%)	3,054 (47.3%)	105 (1.6%)	629 (9.8%)	0	5,251 (81.4%)
2....	25,000	6,650 (26.6%)	40 (0.2%)	14,105 (56.4%)	80 (0.3%)	144 (0.6%)	0	21,019 (84.1%)
3....	330,000	58,231 (17.6%)	137 (0.04%)	177,100 (53.7%)	350 (0.1%)	29,040 (8.8%)	0	264,858 (80.3%)
4....	935,000	540,000 (57.8%)	0	311,500 (33.3%)	0	33,315 (3.6%)	0	844,815 (94.6%)

* The cultures used for the experiments were fed, volume for volume, with inoculated culture fluid at various times prior to the experiment as follows: Experiments Nos. 1, pooled cultures fed 3, 7, 12, 16, and 18 days prior to experiment; 2, pooled cultures fed 2 and 5 days prior to experiment; 3, culture fed 3 days prior to experiment; and 4, culture fed 1 day prior to experiment.

The results are summarized in Table 3. Cultures of various ages were used. It is apparent that the age of the culture influences the distance which the animals will migrate, as evidenced by the fact that, with the 1-day culture, the majority of the animals were found in the first 5-ml. portion withdrawn from the apparatus, whereas with the older cultures the U-tube contained the largest number of animals. Also with the older cultures a higher proportion of dead animals was found, although at the maximum this amounted to only about 2 per cent of the total.

In one case (Expt. 3) a cytological examination of the animals which had not migrated showed that approximately 50 per cent of them were undergoing nuclear reorganization, while less than 10 per cent of the animals in the migrated portion were in autogamy.

Other variations in the design of the migration apparatus are shown in Figures 2 and 3. The apparatus shown in Figure 2 has been used with large mass cultures. Following the initial concentration by filtration through a Berkefeld filter, about 500 ml. of paramecia suspension containing 2,000,000 animals per milliliter is placed in beaker *A*. The anode is placed in position in the bottom of this beaker. A second beaker, *C*, containing not more than 50 ml. of exhausted culture fluid is placed in such a manner that the level of the fluid is exactly at the same height as the level of the fluid in the large beaker. The second electrode is then placed in the beaker, with the end of the agar tube as close to the surface as possible. The end of the tube is surround-

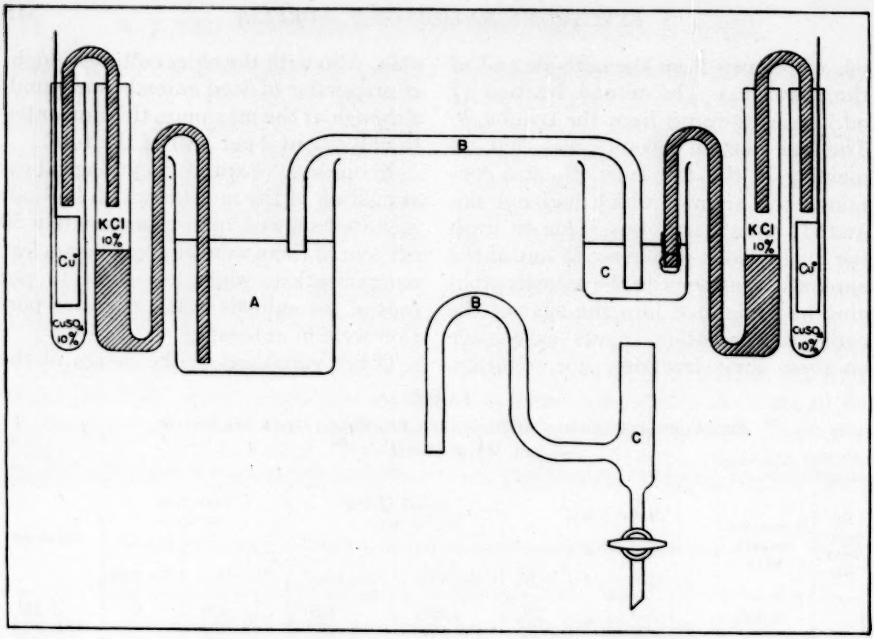

FIG. 2.—Apparatus for use with larger mass cultures

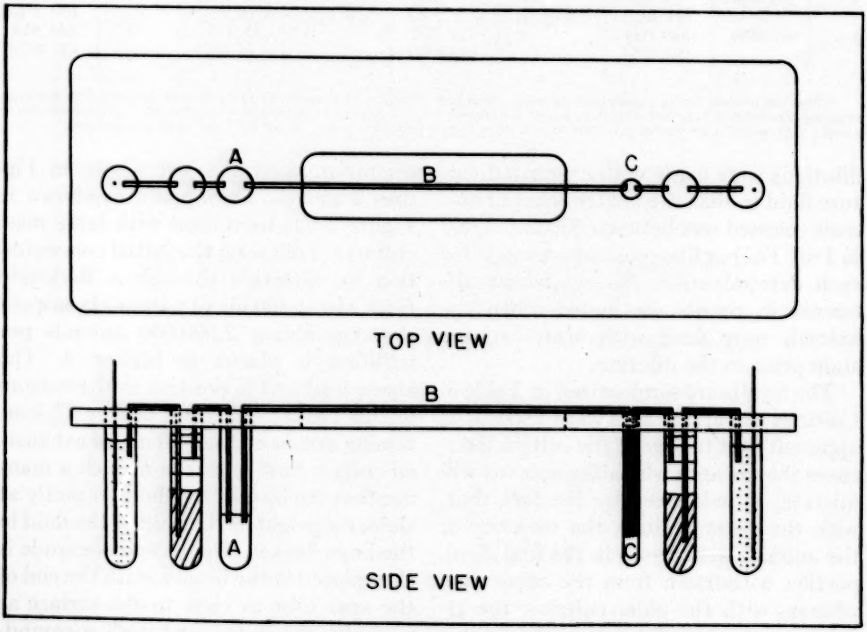

FIG. 3.—Micromodification

ed by a cellophane bag, in orderto prevent leaching of the agar into the migration fluid. A doubly bent tube, *B*, is then filled with medium 2. Each end is closed with a finger, and the tube is inverted and put into the two beakers. If the levels of the fluid in both beakers is at the same height, no movement of the fluids takes place. The migration is usually complete in 4 hours, at which time about 75 per cent of the animals have migrated. The remaining animals can be used to start new mass cultures. It is sometimes advantageous to bubble a small stream of air through the fluid in the large beaker in order to keep the animals well mixed.

A modification which may be used with this apparatus is shown in the lower part of Figure 2. In this apparatus the cathode collection vessel and fluid bridge are constructed as a unit. The cathode is inserted from the top, and the animals collect in high concentration below the cathode in the side tube just above the stopcock. In large mass migrations the paramecia will collect in this side tube and can be withdrawn through the stopcock. Care must be taken that the animals do not remain for long periods of time at a high concentration if live animal suspensions are to be obtained.

The anode-cathode arrangement in Figure 2 can also be used for the electromigration out of large cultures (e.g., 20-liter bottles) by extending the tube containing the agar bridge from the anode so that it will reach the bottom of the bottle. Aeration of the culture is necessary for a satisfactory migration.

A micromodification of the apparatus shown in Figure 3 was used in experiments in which a precisely determined number of paramecia had to be washed and placed in small test tubes. The apparatus is designed for use with a binocular microscope. Small test tubes containing the elctrode solutions, the culture tube, *A*, and the cathode receiving tube, *C*, are mounted in a lucite plate. The migration tube, *B*, and the agar bridges are constructed from 2-mm, thin-walled capillary tubing. The whole apparatus fits on the stage of the microscope with the tubes extending downward on either side of the microscope stage. The stock culture of paramecia is placed in tube *A*. The circuit is closed, and the animals are counted individually as they pass through tube *B*, across the field of the microscope. When the desired number of animals has migrated into the receiving tube, the circuit is opened, the receiving tube is removed, and a new tube is put in its place.

SUMMARY

A description is given of the methods of electromigration which may be used in the concentration and washing of suspensions of *P. aurelia* for use in biochemical and immunochemical studies. Experimental conditions affecting the migrations are reported. Within limits, the speed of migration is affected neither by reducing the concentration of salt used in the migration medium nor by varying the current passing through the apparatus.

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CULTURE VARIATION IN TETRAHYMENA[1]

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RECENT studies on the nutrition of *Tetrahymena geleii* (Elliott, 1949, 1950; Elliott and Hogg, 1949; Hogg and Elliott, 1951) have demonstrated that this ciliated protozoan, when maintained in purified medium, shows definite tendencies to adapt toward a medium deficient in certain nutrilites that have otherwise been considered essential. When attempting to establish the exact nutritional requirements of this animal cell, such culture variation is extremely annoying. However, it must be taken into consideration when any nutritional study is made. Therefore, it seemed important to present information concerning this phenomenon in so far as it has appeared in our present work.

MATERIALS AND METHODS

Tetrahymena geleii, strain E, has been maintained continuously for the last nineteen years in a nutrient broth medium consisting of various substances, which have varied from time to time as new media became available. The most consistently used medium was a 2 per cent solution of Difco tryptone or proteose-peptone with added dextrose (0.1 per cent) and sodium acetate (0.1 per cent) in distilled water. Occasionally a phosphate salt (K_2HPO_4) was added in 0.1 per cent concentration as a buffer. All these substances have been varied, together with others, through the years; but, in general, the organisms thrive on partially degraded casein or beef muscle. Recently, a highly purified (synthetic) medium has become available (Table 1).[2]

When protogen, an unknown growth factor, became available (Stokstad *et al.*, 1949), the culture medium for *T. geleii* was at last reasonably well defined

TABLE 1

BASAL MEDIUM

	γ/Ml.
L-Arginine·HCl	600
L-Histidine·HCl·H_2O	150
DL-Isoleucine	75
L-Leucine	75
L-Lysine·HCl·H_2O	150
DL-Phenylalanine	150
DL-Methionine	150
DL-Serine	300
DL-Threonine	300
L-Tryptophan	150
DL-Valine	150
Dextrose	1,000
Sodium acetate	1,000
$MgSO_4·7H_2O$	100.0
K_2HPO_4	100.0
$CaCl_2·2H_2O$	50.0
$FeCl_3·6H_2O$	1.0
$Fe(NH_4)_2(SO_4)_2·6H_2O$	5.0
$CuCl_2·2H_2O$	5.0
$MnCl_2·4H_2O$	0.05
$ZnCl_2$	0.05
Ca pantothenate	0.10
Nicotinamide	0.10
Pyridoxine·HCl	2.00
Riboflavin	0.10
Pteroyltriglutamic acid	0.01
Thiamine·HCl	1.00
Biotin	0.0005
Choline Cl	1.0
Yeast nucleic acid	100.0
Protogen	1.0 unit

[1] This investigation was supported in part by a grant from the Horace H. Rackham School of Graduate Studies.

[2] The authors are grateful to Merck and Company for supplying the amino acids and vitamins used in this investigation and to Dr. E. L. R. Stokstad for furnishing the protogen.

chemically. The organism has been maintained in this laboratory for the last 2 years in this medium. Serial transfers have been performed once a month. Thus two substrains of this ciliate are available for study, one cultured in nutrient broth for the last nineteen years and a second cultured in a synthetic medium for 2 years. The first we shall refer to as the "peptone strain" and the latter as the "variant strain." The following is a study of the comparative abilities of these two strains to adapt to deficient media.

In general, the techniques have been those employed previously (Elliott, 1949, 1950). Cell suspensions for inoculations were prepared by repeated washings of the cells in distilled water and final dilution to the level which gave a satisfactory initial inoculum to the test cultures (approximately 1,000/ml). Certain precautions were necessary to insure uniformity of inoculations. Each culture tube received 0.2 ml. of a protozoan suspension, delivered rapidly from a 1-ml. pipette. A small pipette was chosen because no appreciable stratification of cells could occur before the suspension was completely discharged. Cultures of 5-ml. volume were grown at 25° C. in selected lipless Pyrex test tubes (15 × 125 mm.). Optical density readings for each culture were made immediately following inoculation and at 24-hour intervals thereafter, employing a Klett-Summerson photoelectric colorimeter (No. 42 blue filter). In general, daily readings were made for 1 week, and the cultures were kept under observation for an additional 2 weeks. Duplicate tubes were inoculated in every test, and, in addition, the duplicates were incubated in separate racks in order to check for any possible environmental variation. Each experiment was repeated several times, and only the most consistent are reported here.

EXPERIMENTAL PROCEDURES AND RESULTS

The nutritional inconsistency of *Tetrahymena* was first noted during the course of a study of the replacement or supplementation of several amino acids by certain structurally or metabolically related compounds (Hogg and Elliott, 1951). It was found that a progressive change of results occurred over a period representing five transfers of the organism in the synthetic medium. Indeed, the variation was so marked that it was necessary to abandon such procedure and to resort to first transfers from a peptone medium—in other words, to utilize a peptone strain exclusively.

In order to determine the extent and nature of this variation, two kinds of tests were instigated: (1) *blank-adaptive growth*, in which growth was determined in the absence of certain essential amino acids, and (2) *substitute-adaptive growth*, in which growth was determined in the presence of a previously nonutilizable amino acid substitute. The amino acids and amino acid substitutes employed are recorded with the results in Figures 1–5.

1. *Blank-adaptive growth.*—Whenever blanks without arginine (Fig. 1) were inoculated with cells that had undergone several (usually five) transfers in synthetic medium, growth appeared after 2 days. Similar blanks seeded with cells from a peptone stock culture failed to show growth after 2 weeks. Growth was not abundant, but it was consistent in several experiments, thus indicating that the organism is able to synthesize arginine slowly.

A similar situation existed when serine (Fig. 2) was withheld from the medium. The organisms from synthetic medium grew to half-maximal levels in 4 days, indicating an even better synthetic ability in regard to this amino acid.

2. *Substitute-adaptive growth.*—In me-

dia without arginine to which citrulline had been added in levels equivalent to the maximal level of arginine in the basal medium, the organisms were able to utilize the substituted compound to such an extent as to produce half-maximal growth (Fig. 3). Apparently citrulline may facilitate the synthesis of arginine in the organisms grown in synthetic media. Cells taken directly from peptone show no evidence of being able to utilize the citrulline at all in the absence of arginine.

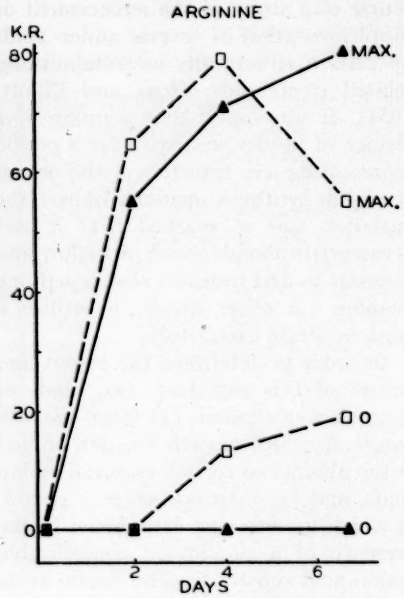

FIG. 1.—Growth of the cells is plotted in Klett-Summerson photoelectric colorimeter readings (*K.R.*) against time in days. Solid lines indicate growth of cells taken from peptone medium (peptone strain), while dashed lines show growth for the variant strain. The zero (*0*) indicates a complete absence of arginine and the *MAX* means that the amino acid was included in its optimal concentration. The same means of identification is employed in Figures 2, 3, 4, and 5.

When arginine was replaced with both ornithine and asparagine, the cells from synthetic media did even better. They attained two-thirds maximal levels of growth (Fig. 4). Again peptone-cultured cells were unable to utilize the substitutes in the synthesis of arginine.

Similarly, α-keto-β-methylvalerate ("ketoisoleucine"), when added to the isoleucine-less medium, brought about two-thirds maximal growth with organisms from synthetic media, while the cultures lacking isoleucine alone grew some, but not well (Fig. 5). Organisms from peptone media were able to grow very slowly (16 days for maximal growth) in the presence of the keto acid substitute (Hogg and Elliott, 1951).

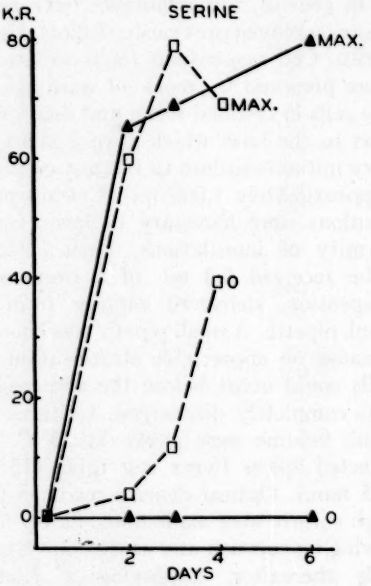

FIG. 2.—Growth is indicated here with and without serine.

Obviously, these data demonstrate that *Tetrahymena* adapts to the lack of certain amino acids and that it can utilize certain related compounds to some extent at least in the place of the missing amino acid. Additionally, it has been noted in several cases that the

variant strain is more responsive to low levels of the amino acids than is the peptone strain, i.e., the variant strain utilizes the amino acids more efficiently.

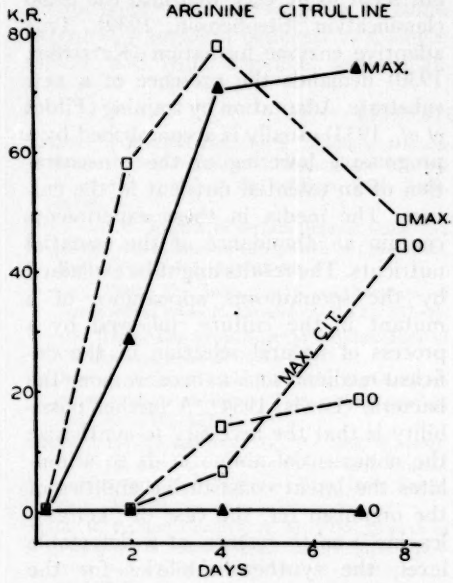

FIG. 3.—Growth is indicated here with and without arginine and also with citrulline substituted for arginine.

DISCUSSION

When strain E was isolated in 1932 (Elliott, 1933), it was noted that, if cells were washed free of bacteria and suspended in a partially hydrolyzed casein medium, growth did not occur. But if the bacteria were withdrawn slowly through several transfers, the Protozoa were finally able to grow satisfactorily in a medium free of other microörganisms. In other words, an "acclimatization period" seemed essential to obtain bacteria-free cultures. The organisms seemed to require a period of time to adapt to a nonparticulate diet. Furthermore, when this cell was first isolated, it seemed to grow best on proteins or partially hydrolyzed proteins, such as casein, but later it seemed to do better when high concentrations of amino acids were present in the medium (Elliott, 1935; Hall and Elliott, 1935). More recent work (Elliott, 1949) has demonstrated that it can thrive with pure amino acids and nucleic acid as the sole nitrogen sources. This entire picture seems to indicate that the organism has been able to adapt to a simpler nitrogen diet through the years. The present work demonstrates that the adaptation is continuing in the synthetic medium, even to the point of getting along without certain of the amino acids that were thought to be essential a year or so ago. The question arises as to how far this ability to adapt can be forced.

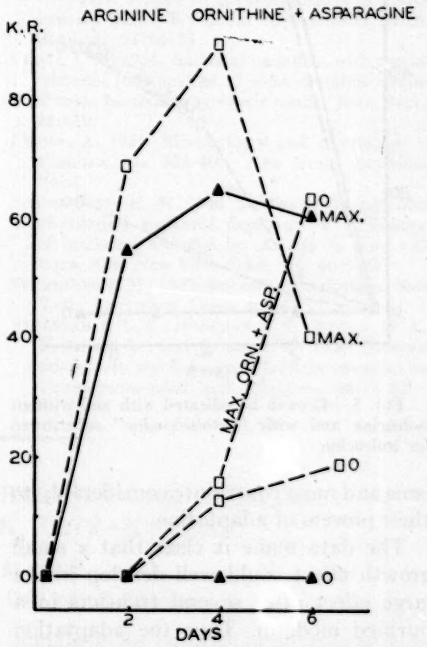

FIG. 4.—Growth is indicated here with and without arginine and with a mixture of ornithine and asparagine substituted for arginine.

It has been noted during this work that the Protozoa retain their viability in the various deficient media, even though no growth occurs. Thus addition of the lacking nutrient after 3 weeks' time will usually produce a normal growth response. This maintenance of viability in amino acid–deficient media is a remarkable feature of these organ-

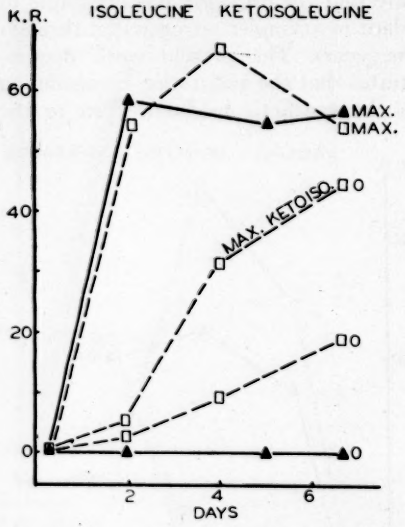

FIG. 5.—Growth is indicated with and without isoleucine and with "ketoisoleucine" substituted for isoleucine.

isms and must contribute considerably to their powers of adaptation.

The data make it clear that a small growth effect could well develop into a large effect after several transfers in a purified medium. Thus the adaptation phenomenon may well explain the difference in nutritional requirements of various strains of *T. geleii* which have been studied. Particular cases that may well be explained on this basis are the contradictory reports on the requirements of *T. geleii* for serine (Kidder and Dewey, 1947; Elliott, 1949) and for riboflavin (Kidder and Dewey, 1949; Elliott, 1950). Certainly this phenomenon should be considered before any dogmatic statements are made (Lwoff, 1951).

This adaptation phenomenon does not seem to fit very well into the usual classification (Stephenson, 1939). True adaptive enzyme formation (Karström, 1930) demands the presence of a new substrate. Adaptation by training (Fildes *et al.*, 1933) usually is accomplished by a progressive lowering of the concentration of an essential nutrient in the culture. The media in these experiments contain an abundance of the essential nutrients. The results might be explained by the spontaneous appearance of a mutant in the culture, followed by a process of natural selection in the deficient medium, such as occurs among the bacteria (Lewis, 1934). A further possibility is that the necessity to synthesize the nonessential amino acids so stimulates the latent constitutive abilities of the organism (cf. the case of "ketoisoleucine") as to restore, at a detectable level, the synthetic abilities for the essential amino acids.

Schoenborn (1940) noted an adaptation phenomenon occurring when pure cultures of the flagellates, *Astasia sp.* and *Euglena gracilis*, were transferred from peptone to simple media containing only an inorganic source of nitrogen. He called this a process of adaptation involving selection. We may be seeing a similar situation with *Tetrahymena*. Whatever the nature of the adaptation may be, it still provides a convenient experimental tool for the study of biosynthetic processes.

SUMMARY

A culture variation of *T. geleii* in synthetic medium has been observed in several instances. These variations have been characterized with respect to the nutritional requirements for certain

amino acids. Two experimental types were observed: (1) blank-adaptive growth and (2) substitute-adaptive growth. In type 1 the organisms grown in synthetic media were observed to grow to some extent in a medium deficient in a single amino acid (e.g., arginine, serine). In type 2 the variant organisms grew in the presence of previously nonutilizable amino acid substitutes (e.g., citrulline, "ketoisoleucine"). Several possible explanations are considered, and the significance of the phenomenon for research on *Tetrahymena* is emphasized.

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THE INFLUENCE OF COBALT ON THE GROWTH OF THE PROTOZOAN, TETRAHYMENA[1]

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IN THE evolution of protoplasm, many metal ions were incorporated into the living mass, and a few have been found to be indispensable. The transition metals of the periodic table appear to be particularly important. The studies made independently by Rickes *et al.* (1948) in this country and Smith (1948) in England first demonstrated that vitamin B_{12} contained cobalt. The only report in the literature on *Tetrahymena* mentioning the effect of this metal on growth is that of Kidder *et al.* (1951), in which negative results were reported. Elliott (1950) re-

[1] My appreciation is extended to my wife, Nina Medlyn Slater, for aid during this experimentation.

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ported that vitamin B_{12} had no effect on strain E.

The present-day experimenter, interested in finding out something about the biological effects of cobalt, is instantly impressed with the desirability of eliminating extraneous sources of this metal from nearly everything involved in the experiment. This is laborious and uncertain, and the biologist must often be content with a reduction to very low levels but not to an absolute removal of the trace metal. Also, considering the complexity of protoplasm, it is nearly impossible to remove any metal from the intracellular milieu without damage to the growth process. Living matter stores certain metal ions, and therefore the initial inocula may be a source of contamination. This is especially true if some cells of the inoculum die and release enough of their contained metabolites for the initiation and continuation of growth of the survivors. The very low requirement for cobalt in most instances implies that a slight supply from this source could be highly significant. Of course, if no growth at all occurs in the supposed absence of a particular material, the environment may be considered sufficiently pure in regard to that factor. As Foster (1949) has said, the biological test, in the final analysis, is the most critical criterion of purity.

It was previously stated (Slater, 1952*a*) that the purification of all the ingredients in the diet of *Tetrahymena* was a major and difficult task. With a combination of methods, involving chelation and the use of highly purified salts, it has been possible, nonetheless, to demonstrate the essentiality of cobalt for the growth of this ciliate. The term "essential" seems to have had different usages in the literature. In this paper it is taken to mean that, in the absence of the test material, *no growth whatsoever* was obtained.

Spectographic analysis can be used to evaluate purity of media. Even this, though the most critically sensitive method available to the physical scientist, may fail to detect small amounts of a cation which may be quite significant physiologically.

MATERIALS AND METHODS

The animal employed in this investigation was the ciliate, *Tetrahymena geleii* E. Culture and general experimental conditions are those previously described (Slater, 1952*a*). Great care was taken to avoid dust in the experimental vessels. All glassware was of Pyrex brand. Stout and Arnon (1939) reported that Pyrex containers gave good results in other investigations on microelements and that this kind of glass was not a source of metal contamination. Glassware was washed with a detergent, rinsed with at least eight changes of hot running tap water to remove the detergent, and then soaked in distilled water for ½ hour. Two more distilled-water rinses were used after this. All tubes were then rinsed with two washes of glass-distilled water and, finally, while three-quarters filled with glass-distilled water, autoclaved for 1 hour. They were then drained while yet hot and dried in a hot-air sterilizer. This autoclaving was reported by Waring and Werkman (1942) to be necessary for the removal of adherent iron and conceivably any cobalt which also might adhere in this fashion. In two series of tests where this autoclaving was omitted, results were unaffected, however. All the water used in the construction of media and in the final washes of glassware was slowly distilled by means of an all-Pyrex glass still. Glass-distilled water was percolated for at least 30 minutes through all the pipettes after they had received

the customary distilled-water rinses. This was accomplished by placing the pipettes in a 2-liter Erlenmeyer flask about half-filled with glass-distilled water and then boiling the water. The pipettes were then sterilized by means of dry heat while wrapped in several layers of glass wool in pipette cans. All other glassware was carefully rinsed twice with boiling distilled water after the usual rinses. It was found that glass wool plugs in the test tubes did not give different results than were obtained by the use of cotton plugs.

Growth was measured turbidimetrically by means of the Lumetron photoelectric colorimeter (Model 401) in which the red filter (650 mμ) was used. The test substrate employed (Table 1) is modified from Elliott (1949, 1950).

The amino acids, nucleotides, pyrimidine, phosphate, and acetate were dissolved in glass-distilled water and treated with 8-hydroxyquinoline (oxine) for the removal of trace metals by the method of Chesters and Rolinson (1951*a*). The chelator was removed with chloroform. After removal of most of the chloroform by means of a separatory funnel, the rest was boiled out of the media. Final traces were removed during autoclaving.

The growth factors were not subjected to the purification process, since they were introduced in such small amounts as to make metal-ion contamination extremely slight. It was unnecessary further to purify the Johnson, Matthey "spec-pure" salts. The Mn and Zn salts were used as the analytic grade, since they were unavailable otherwise. These two salts were used in such low concentration as to make accompanying trace metals of slight magnitude. It was necessary to dissolve the $CaCO_3$ in concentrated HCl prior to the introduction of this salt into the medium. The nucleotides and pyrimidine were first dissolved in hot 0.1 N sodium hydroxide. The pH was adjusted to 7.5, and incubation was in the dark at 25° C.

In preliminary experiments, glucose which was subjected to the purification procedure did not alter results obtained when analytic-grade glucose was used without purification. Subsequent work therefore involved the use of this carbohydrate without purification. The cobalt salt used was spectrographically purified $Co(NO_3)_2 \cdot 6H_2O$, and the concentrations used were those shown in Figure 4.

Cori *et al.* (1938) showed that cobalt

TABLE 1

BASAL MEDIUM USED IN GROWTH STUDIES ON *Tetrahymena geleii* E.

Substance	γ/Ml
L-Arginine monohydrochloride	600
L-Histidine monohydrochloride	150
DL-Isoleucine	75
DL-Leucine	75
L-Lysine monohydrochloride	150
DL-Methionine	150
DL-Phenylalanine	150
DL-Serine	300
DL-Threonine	300
DL-Tryptophan	150
DL-Valine	150
Glucose	1,000
$NaC_2H_3O_2 \cdot 3H_2O$	1,000
Adenylic acid	25
Cytidylic acid	25
Guanylic acid	25
Uracil	25
Calcium pantothenate	0.10
Nicotinamide	0.10
Pyridoxine hydrochloride	2.00
Riboflavin	0.10
Folic acid	0.01
Thiamine hydrochloride	1.00
α-Lipoic acid	0.05
KH_2PO_4	100.00
$CaCO_3$*	50.00
$MgSO_4 \cdot 7H_2O$*	10.00
$FeSO_4 \cdot 7H_2O$*	5.00
$CuSO_4 \cdot 5H_2O$*	5.00
$MnCl_2 \cdot 4H_2O$	0.50
$Zn(NO_3)_2 \cdot 6H_2O$	0.05

* Johnson, Matthey & Co. (London, England), spectrographically standardized. No lines for cobalt were detected in any of these salts.

was important in the action of phosphoglucomutase, and in 1949 Speck showed that oxalacetate was activated by cobalt. Therefore, since Elliott and Hunter (1951) have presented evidence for phosphatase activity in *Tetrahymena* and Seaman (1949) has reported the presence of the tricarboxylic acid cycle in this ciliate, it was decided to study the possible effects of cobalt in respect to the amount of glucose substrate.

All tests and controls were undertaken in duplicate tubes, and the results are the averages from five experiments in which highly purified α-lipoic acid was used. This new growth factor was isolated by Reed *et al.* in 1951. It has considerable acetate-replacing properties for *Tetrahymena* and can replace protogen as an essential growth factor for *Tetrahymena*, according to Slater (1952*b*) and Seaman (1952).

Two series of experiments in which protogen B was used instead of α-lipoic acid gave identical results. The empirical formula of this latter factor has recently been published as $C_8H_{14}S_2O_2$ (Reed, 1952), and it is stated to be a monocarboxylic acid.

Protogen B was supplied as a highly purified concentrate (Stokstad *et al.*, 1952) and is metal-free (Stokstad, 1952). The author is indebted to L. J. Reed, of the University of Texas, for concentrates of α-lipoic acid and gratefully acknowledges the aid of E. L. R. Stokstad, of the Lederle Laboratories Division, American Cyanamid Company, in supplying generous quantities of protogen. Thanks are also due S. H. Hutner for a critical examination of the manuscript and for many helpful suggestions.

RESULTS

Tetrahymena did not grow at all in purified, glucose-free media unless at least 0.5 γ/ml of cobaltous ion was present (Fig. 1). Until populations were close to the end of their growth capacity, levels of cobalt as high as 8 γ/ml resulted in a slight but consistently lower amount of growth than obtained with 1–4 γ/ml, and then the higher concentration of cobalt resulted in a slightly greater peak. This higher amount of metal ion caused a 24-hour increase in lag phase over cultures with 1–4 γ/ml of the ion. At the end of 9 days' incubation, concentrations of cobalt of from 1 to 8 γ/ml produced about the same amount of growth (Fig. 2). Concentrations of cobalt of 0.5 γ/ml, in the absence of glucose, resulted in an extremely long lag phase; but, upon the initiation of active and measurable growth, the rate was the same as with the other concentrations. Peak growth at this low level was about 75 per cent of that obtained when optimal amounts of cobalt were present (1–4 γ/ml). Although three extra rinses of chloroform were used in the purification process in order to remove the last traces of oxine,

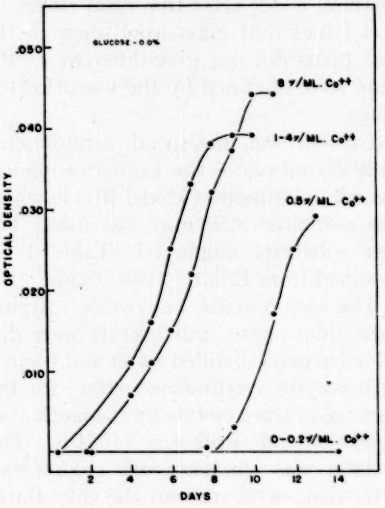

FIG. 1.—Effect of cobalt on the growth of *Tetrahymena* in glucose-free media.

the possibility existed that some of the chelator might still have remained in solution. It was therefore decided to attempt to inactivate this material and thus presumably to initiate growth in glucose-free media in the absence of cobalt. Copper added for this purpose did not initiate growth, however,

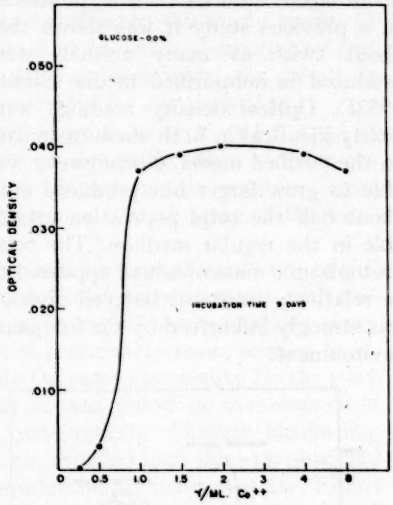

FIG. 2.—Dose response of *Tetrahymena* to cobalt in glucose-free media.

In media containing 0.025 per cent glucose, the ciliate was able to adapt to an environment in which cobalt was either absent or present to an extremely low degree. The lag phase under these circumstances was much longer than that obtained when the highest concentration of the trace metal was added (Fig. 3). Growth rates and peak growths were not affected. Until these cultures had grown for about 4 days, it seemed that cobalt was as indispensable under these conditions as when cultures were grown in the absence of the carbohydrate (Fig. 4); but, after 9 days, all these cultures had reached the same peaks. The question arises as to whether the glucose was contaminated with cobalt. In order to have enough cobalt present for any growth response at all, as seen when glucose was omitted from the media (0.5 γ/ml), would imply that the carbohydrate would have to carry at least 0.2 per cent cobalt as impurity. The analysis supplied with this carbohydrate indicated that all the accompanying impurities together did not amount to more than 0.02 per cent; in any case, it is extremely unlikely that this would be entirely cobalt.

In media with the full complement of glucose (0.1 per cent), *Tetrahymena* grew consistently better in the presence of 4–8 γ/ml of cobaltous ion as compared with populations started in media containing less cobalt than this. At the end of 10 days' incubation, the peaks were all of the same magnitude regardless of the amount of trace metal present.

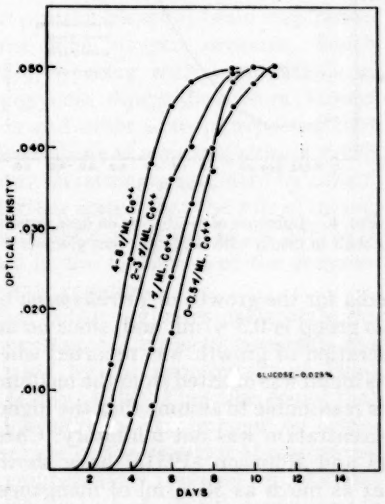

FIG. 3.—Effect of cobalt on the growth of *Tetrahymena* in media with 0.025 per cent glucose.

The growth rates of cultures subjected to the chelation procedure were about two-thirds that obtained when the regular media were used (Fig. 5). The purified

media contained 0.5 γ/ml of manganese chloride as compared with 0.05 γ/ml in the usual media (Slater, 1952*b*). It was reported by Kidder *et al.* in 1951 that manganese was not required by strain W. The usual amount of manganese used in

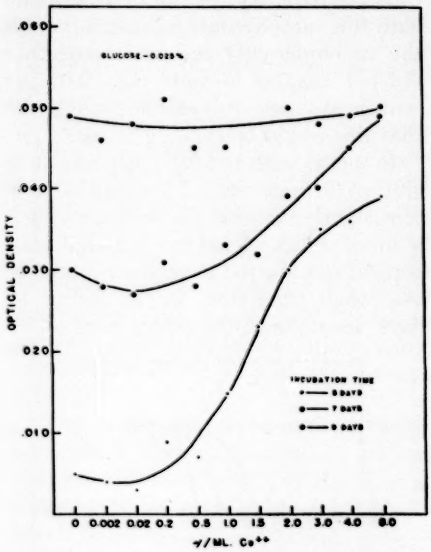

FIG. 4.—Influence of incubation on dose response to cobalt in media with 0.025 per cent glucose.

media for the growth of *Tetrahymena* by this group is 0.5 γ/ml, and, since no acceleration of growth was reported when this metal was omitted from the medium, it is reasonable to assume that the higher concentration was not inhibitory. Chesters and Rolinson (1951*b*) have shown that as much as 50 γ/ml of manganese had little effect on the growth of *Streptomyces griseus*. Of course, though, manganese at the 0.5 γ/ml level may have been inhibitory for *Tetrahymena* in purified media. It is believed, however, that the very much reduced rate of growth in the purified media was due to either a lack of some other inorganic substance or to an inhibiting cation which became unmasked when an antagonistic ion was removed.

Cell counts made at the end of the growth phase in purified media containing 0.1 per cent glucose and either zero or 8 γ/ml of cobaltous ion showed that only about 60,000 animals/ml were produced. In a previous study it was shown that about twice as many animals were produced in nonpurified media (Slater, 1952*b*). Optical density readings were nearly identical in both these instances. In the purified media, *Tetrahymena* was able to grow larger but produced only about half the total population attainable in the regular medium. The total protoplasmic mass obtained appeared to be relatively constant, but cell division was strongly influenced by the inorganic environment.

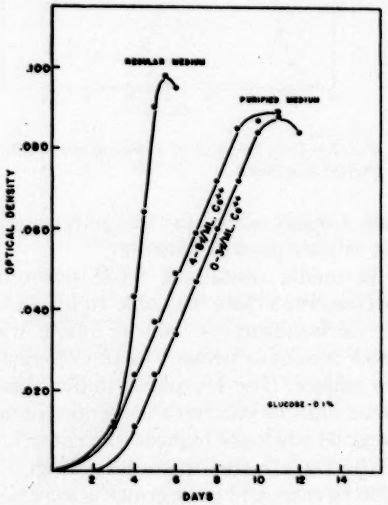

FIG. 5.—Influence of purification of the medium on growth rate.

DISCUSSION

Cobalt has been known to be present in protoplasm for many years and at first was believed to be of fortuitous origin. Marston's review (1952) reveals the vast

amount of work which has been done on this trace metal since the discovery that it was an essential component in the diet of ruminants. Marston also states that all attempts to show that this metal per se is of importance to animals other than cattle and sheep have failed. The experiments described in this study appear to be the first in which a qualitative and quantitative essentiality for cobalt is demonstrated for any protozoan.

In this work, as little as 0.5 γ/ml of cobaltous ion produced a definite response, which puts this metal in a class analogous to that of some of the vitamins.

The first few days of incubation of cultures of *Tetrahymena* in media with glucose and very low concentrations of cobalt, or presumably none, seemed to indicate the same essentiality for the trace metal as was found in the absence of the carbohydrate. Further incubation, though, revealed that this organism had a considerable adaptive capacity. Elliott and Hogg (1952) have demonstrated that, in several instances, strain E will eventually grow when supplied with an initially unavailable substrate. According to Catcheside (1951), in adaptation a population of cells placed in contact with a substrate acquires after a time the enzyme necessary to metabolize the substrate, and a removal of the substrate leads to the disappearance of the enzyme system it had evoked. It is not known whether this enzyme disappearance would occur in *Tetrahymena* under the circumstances described here.

Vitamin B_{12} appears to have several metabolic functions. E. L. Smith (1951) pointed out that both B_{12} and folic acid play some part in the synthesis and metabolism of labile methyl groups and possibly other one-carbon fragments. Smith also stated that B_{12} appears to have a lipotropic action in rats and may help in the utilization of large amounts of protein. The work of Jukes *et al.* (1950) suggested that vitamin B_{12} controlled the synthesis of the desoxyribosides of guanine, hypoxanthine, adenine, and cytosine.

Marston (1952) suggested that the prime function of cobalt in the animal organism was to serve as a part of vitamin B_{12}. There is a great deal of literature, however, on the importance of this trace metal per se as an enzyme activator. Punt (1942) reported that cholinesterase activity was enhanced by the use of cobalt, and Johnson and Berger (1942) showed that cobaltous ion was an exceedingly specific activator of glycylglycine hydrolysis. The experiments by Dickens (1946) on the toxic effects of oxygen on brain metabolism showed that cobalt could protect (*in vitro*) brain respiration against high oxygen pressure. Smith (1948*b*), working with preparations of glycylglycine dipeptidase from human uterus and other sources, reported that the hydrolysis of sarcosylglycine by this enzyme was strongly activated by cobalt. He further stated that the role of metals in hydrolytic reactions is to act as a bridge in the formation of the enzyme-substrate complex.

That cobalt activates oxalacetate decarboxylase of *Azotobacter vinelandii* was indicated by the experiments of Plaut and Hardy (1949). They also found that the inhibition of this enzyme by orthophosphate in the presence of manganese was competitively reversed by cobaltous ion. Hearon *et al.* (1949) demonstrated that histidine and cobalt form a complex and are able to combine reversibly with molecular oxygen. *Tetrahymena*, in a partially anaerobic environment, might conceivably receive a part of its oxygen supply in this manner.

Magnetic susceptibility measurements made by Diehl *et al.* (1950) indicate that

the cobalt in B_{12} is either in a trivalent state or in a bivalent state coupled with molecular oxygen. If this metal is in the trivalent form, a proper comparison of any salt of cobalt with the vitamin should involve the use of the cobaltic ion. The divalent ion has been used in most of the experimental work.

Becker *et al.* (1949) in experiments with cobalt-deficient lambs showed that there was no significant response to injections of B_{12} in amounts as high as 125 μgm. Marston (1952) has, since this, reported that when as much as 300 μgm. of B_{12} are injected per week into cobalt-deficient sheep, both the blood dyscrasia and the general condition responded more rapidly than to dosing with 1,000 μgm. of cobalt per day. He concluded that there is little doubt that the syndrome of cobalt deficiency in sheep is the result of a profound vitamin B_{12} deficit. Hendlin and Ruger (1950) demonstrated that cobalt could be a limiting factor in the biosynthesis of B_{12} by certain bacteria.

In 1949, Hutner *et al.* described the use of the phytoflagellate *Euglena gracilis* for the assay of vitamin B_{12}. Later, Hutner *et al.* (1950) stated that this organism required 4,900 molecules of B_{12} for the formation of one cell and that the actual amount of cobalt needed was about 6×10^{-13} gm/ml of culture medium. Hutner and Provasoli (1951) added that *E. gracilis* has shown little ability to utilize B_{12} when in high molecular combination.

Tetrahymena can utilize certain conjugates of folic acid, as shown by Kidder and Dewey (1947), and might be able to break down B_{12} because of this ability to attack large molecules. On the other hand, *Tetrahymena* might manufacture B_{12}. This vitamin can replace the purines in the nutrition of *L. lactis*, as described by Shive *et al.* (1948), but whether this can occur in *Tetrahymena* has not been determined.

Although it appears from the experiments reported here that the organic environment influences the inorganic requirement for cobalt, it seems likely that various inorganic ions may also influence any particular trace-metal essentiality or activity at the enzymatic level.

Lehninger (1950) in a review examined the role of metal ions in enzyme systems and discussed a number of approaches to the problem of the physical basis of metal-ion specificity. He added that the complex formation which is possible between metallic ions and certain anions, as $HPO_4^{=}$ and $SO_4^{=}$, is not sufficiently appreciated by the biologist.

MacLeod and Snell (1947) demonstrated that the manganese requirement in certain bacteria was decreased by additional amounts of magnesium. They also showed (1950) that zinc toxicity in *L. arabinosus* could be completely released by means of manganese, magnesium, or calcium. The requirement of this organism for divalent manganese was also decreased by magnesium, calcium, or strontium. These sparing effects on the nutritional requirement for essential ions were interpreted to mean that these related ions carried out some but not all of the catalytic roles served by the corresponding essential ions. The zinc toxicity was thought to be caused by the formation of a catalytically inactive zinc-protein, and it was suggested that counteraction of this by means of manganese, magnesium, calcium, or strontium resulted because of the displacement of the zinc and the subsequent formation of a catalytically active metalloprotein. Abelson and Aldous (1950) demonstrated with *E. coli* that the toxicity of cobalt and nickel for this bacterium was significantly lowered by high amounts of magnesium and that the amounts of cobalt

and nickel actually bound by the cell were markedly decreased. An adverse response of the young rat to a diet high in zinc was demonstrated by Smith and Larson (1946) to be reversible with copper and iron.

The inorganic ion interrelationships in the growth of *Tetrahymena* are unexplored at present, and fruitful results can be expected from work in this direction.

Until the ingredients in the basal medium for *Tetrahymena* can all be obtained commercially and with guaranteed purity, the construction of experiments for the evaluation of the physiological activity and growth effects of the inorganic environment will continue to be somewhat laborious. At the present, a combination of the chelation technique and the use of inorganic salts of known purity hold much promise for future researches with this animal.

SUMMARY

1. Cobalt is essential for the growth of *Tetrahymena* in purified, glucose-free medium.

2. As little as 0.5 γ/ml of cobaltous ion produced a definite response in the absence of glucose. The greatest amount of growth was obtained with 8 γ/ml of this ion, and 1–4 γ/ml resulted in slightly less growth. The lag phase was much prolonged when only 0.5 γ/ml of the trace metal was added.

3. Maximal growth, in media with 0.025 per cent glucose, was obtained even when cobalt was not added, but the lag phase of populations in this environment was more than 50 hours longer than found when 4–8 γ/ml of cobaltous ion were present.

4. Cultures in media containing 0.1 per cent glucose reached maximal growth at the same time, regardless of the amount of cobalt present, but cultures in media containing 0–3 γ/ml of the trace metal exhibited a lag phase which was 24 hours longer than obtained when the media contained 4–8 γ/ml of cobalt.

5. Cultures in purified media with 0.1 per cent glucose reached the same peaks as cultures in nonpurified media, but the growth rate was only two-thirds that obtained with the regular media. The inorganic environment is considered responsible for this.

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THE MODIFICATION OF DEVELOPMENTAL PATTERNS IN THE SAND DOLLAR BY SODIUM SELENITE[1]

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EXPERIMENTS dealing with the modification of developmental pattern in the echinoderm egg by external agents are not new. Sixty years ago Herbst (1892) discovered that lithium would cause exogastrulation and vegetalization in the developing larva of the sea urchin. Child (1941*a*, pp. 197–246) reviewed the various agents that affect development differentially and pointed out that differential inhibition, differential recovery, and differential conditioning in a quantitatively graded physiological system may be largely responsible for modifications in development caused by external agents.

Recently, others (Hörstadius and Gustafson, 1947; see Hörstadius, 1949, for review) have found that certain substances (propanediol phosphate, phosphogluconic acid, lactate), which are thought to play a role in carbohydrate metabolism, cause an animalization in apical halves of the sea-urchin egg, while other substances (certain amino acids), which may tend to favor protein metabolism, cause vegetalization. Also recently, the author (Rulon, 1949; 1950*a*, *b*; 1951) has shown that in the developing egg of the sand dollar (*Dendraster excentricus*) certain enzyme inhibitors (azide, thiourea) promote vegetalization, while others (maleic acid, malonic acid) promote overdevelopment of the animal region. The different inhibitors also have other dissimilar effects. In continuation of this work and in an effort to learn more concerning the dynamics of development, the action of sodium selenite, an agent reputed to have little effect on cytochrome oxidase activity but definitely inhibitory to succinic dehydrogenase (University of Wisconsin Biochemists, 1939; Bernheim, 1942), was followed when applied to different developmental stages.

MATERIAL AND METHODS

As in most of the author's previous work on the effects of enzyme inhibitors on morphogenesis in echinoderms, the eggs of the sand dollar, *D. excentricus*, were used. The adults of this species were dredged from Monterey Bay during the breeding season (June–August) and kept in running sea water in the aquarium room of the Hopkins Marine Station. All experiments were conducted and all material was kept in this room, where the temperature varied by no more than 1° from 18° C. The large clear eggs were taken from ripe females, washed in sea water, and fertilized by adding a few drops of sperm suspension. During the course of these experiments fertilization was almost 100 per cent. Test and control eggs were kept in covered finger bowls at room temperature. The test solution (Na-selenite) was made up at M/100 strength in sea water. It was diluted with sea water to fit experimental requirements. Smoking was not per-

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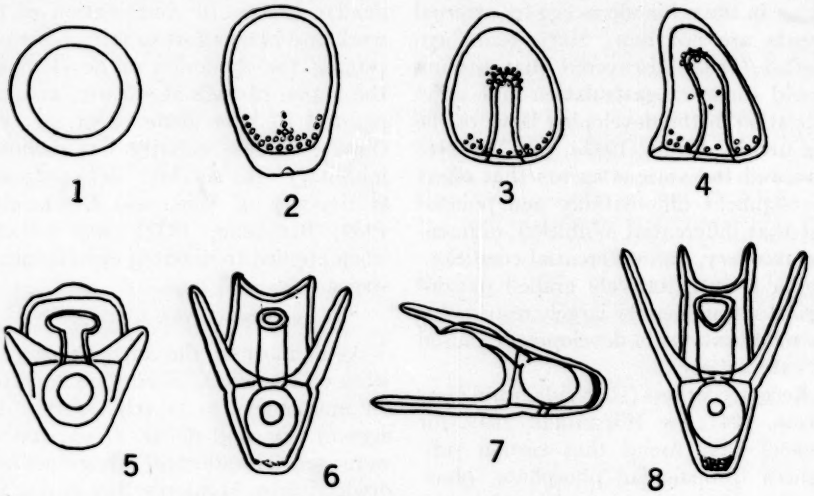
mitted in the laboratory. Preliminary experiments indicated that M/200 Na-selenite was near the limit of tolerance and that the most effective range was from M/200 to M/3,200.

NORMAL DEVELOPMENT

At approximately 18° C. and under laboratory conditions the fertilized egg of *Dendraster* reaches the mid-blastula stage (Fig. 1) by the end of 6 hours. At this stage the walls are thick but smooth, the fertilization membrane is still intact, and ciliary movement has not yet begun. At 12 hours the larva has become ciliated, it has broken out of its fertilization membrane and is usually actively swimming near the surface of the culture. The walls, except for the vegetal region, have thinned, primarily mesenchyme has appeared, and there may be slight invagination (Fig. 2). By 18 hours the gut has invaginated, ventrodorsality has appeared, and the apical end has become secondarily thickened (Fig. 3). At 24 hours the prepluteus larva has a flattened ventral surface, an enteron that is large and directed toward the future stomodeal region, and rudiments of anal arms appearing in basal-lateral positions of the ventral surface (Fig. 4). At 30 hours the ventral surface is concave, the coelomic pouches are present, the oral lobe is taking shape, and the skeletal spicules are forming rapidly (Fig. 5). At 42 hours the stomodaeum opens into the gut, the ventral surface is deeply concave, the anal arms are fairly well developed, the oral lobe has decreased in breadth, and the oral arms are just beginning to appear (Fig. 6). The 60-hour pluteus is well developed, with long oral and anal arms (Figs. 7 and 8).



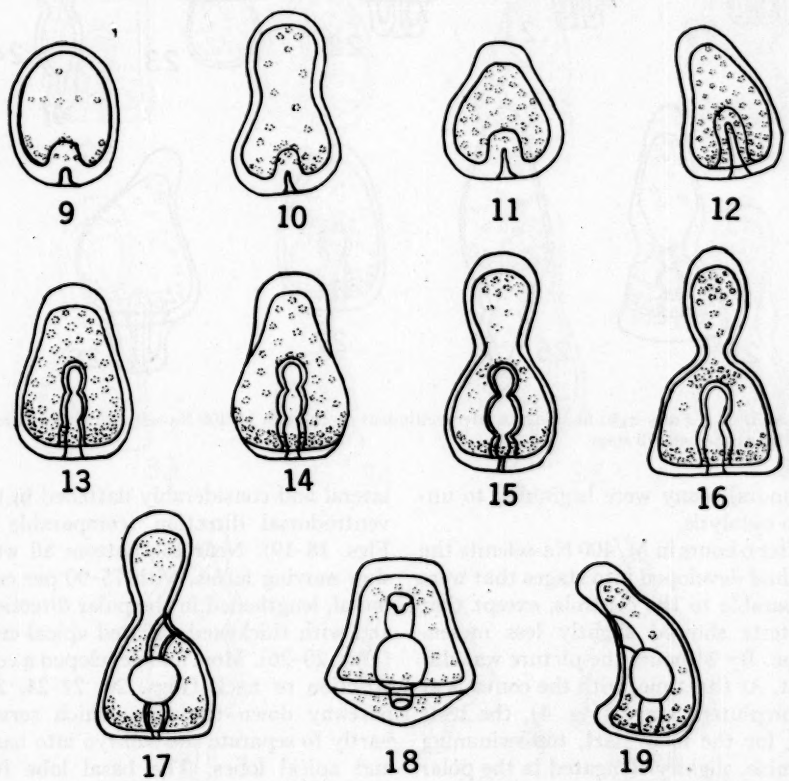
FIGS. 1–8.—Normal development of the sand-dollar larva from 6 to 60 hours at a temperature of approximately 18° C.

EXPERIMENTAL

I. CONTINUOUS EXPOSURE TO NA-SELENITE BEGINNING WITH THE ONE-CELL STAGE

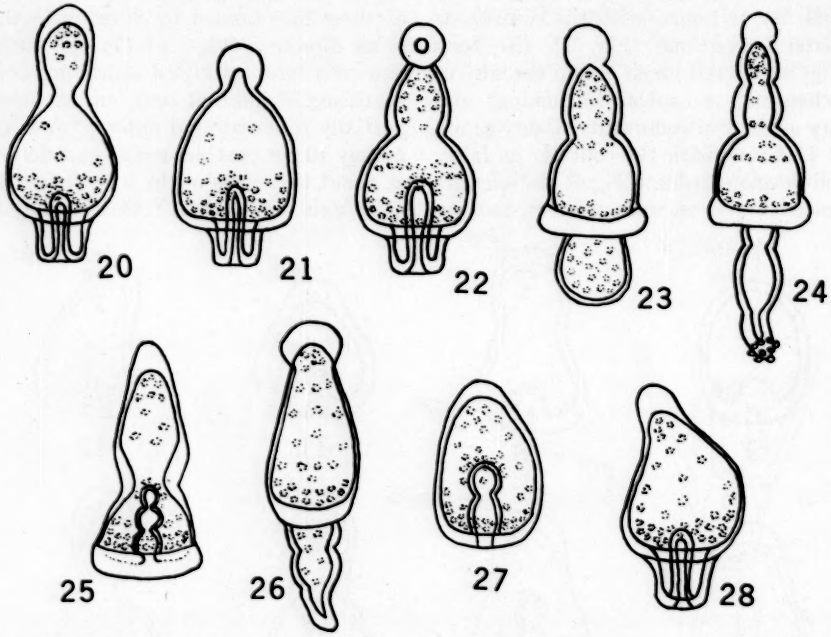
Fertilized eggs, placed in a sea-water solution of M/200 Na-selenite, were only slightly behind the controls after 6 hours'

exposure (see Fig. 1 for 6-hour blastula). With 12 hours' exposure, when the controls had reached the thin-walled, free-swimming, late blastula stage (Fig. 2), the treated forms, called here the "tests," appeared as mesenchyme-free blastulae with walls slightly thicker than the controls. By 18 hours, with the controls as bilateral gastrulae (Fig. 3), the tests (Fig. 9) showed much less in the way of archentera, no apical thickenings and very slight ventrodorsality, if any at all. At 42 hours, with the controls as fairly well-developed plutei (Fig. 6), the selenite-treated embryos were all slow-moving bottom forms (Figs. 10–12), with most having small archentera and apical thickenings. All the tests had an excess of internal cells, and only approximately 50 per cent had developed bilaterality (Fig. 12). By 52 hours, approximately half had remained radial in symmetry, and most of these had tended to elongate in the polar direction (Figs. 13–17). All those observed had thickened apical regions, an excess of internal cells, and at least partially differentiated entera. Approximately 10 per cent showed a constriction at about two-fifths of the way down the polar axis (Figs. 15–17). Occasionally a



FIGS. 9–19.—Larvae resulting from continuous exposure to M/200 Na-selenite, with treatment beginning at the one-cell stage. Fig. 9, after 18 hours in the test solution. Figs. 10–12, after 42 hours. Figs. 13–19, after 52 hours.

stomodaeum developed slightly below this constriction. The remaining eggs (approximately 50 per cent) developed flattened, bilateral forms, without skeleton, and with a ciliated band about the ventral area (Figs. 18–19). By this time traces of ventrodorsality (as Fig. 12). After 47–48 hours most of the test embryos had dropped to the bottoms of the culture dishes, leaving less than 5 per cent of the total near the surface. These free-swimming forms were usually bi-



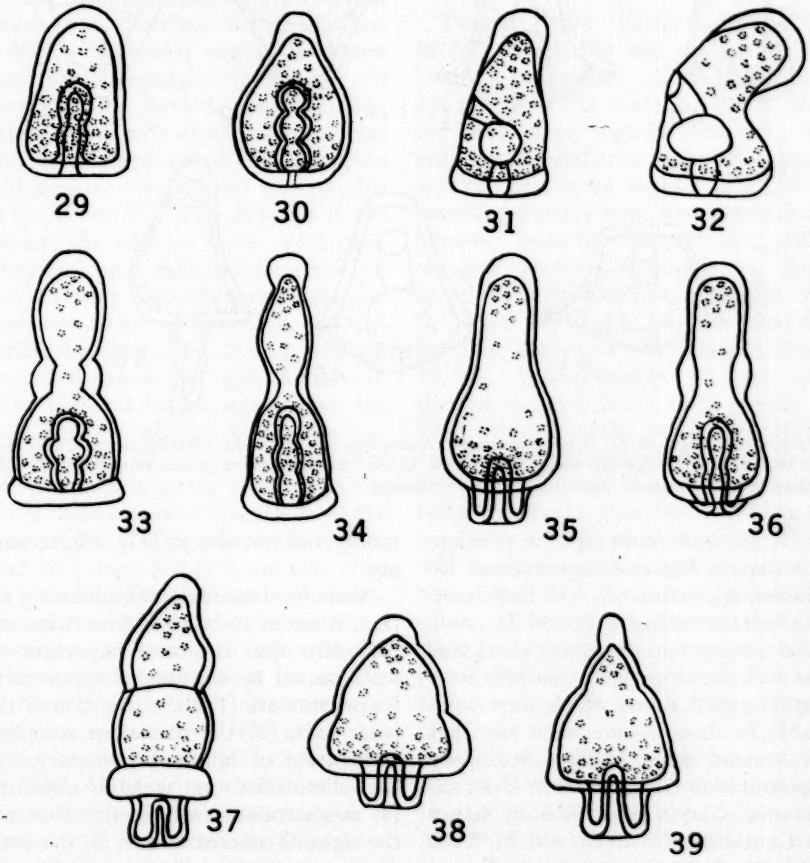
FIGS. 20–28.—Forty-eight-hour larvae after continuous exposure to M/400 Na-selenite, with treatment beginning at the one-cell stage.

(52 hours) many were beginning to undergo cytolysis.

After 6 hours in M/400 Na-selenite the eggs had developed into stages that were comparable to the controls, except that the tests showed slightly less mesenchyme. By 24 hours the picture was different. At this time, with the controls at the prepluteus stage (Fig. 4), the tests were, for the most part, top-swimming gastrulae, slightly elongated in the polar direction. Over 50 per cent were definitely radial (comparable to Fig. 10 in appearance), with the remainder showing lateral and considerably flattened in the ventrodorsal direction (comparable to Figs. 18–19). Near the bottom all were slow-moving forms, with 75–90 per cent radial, lengthened in the polar direction, and with thickened, ciliated apical ends (Figs. 20–26). Most had developed a constriction or neck (Figs. 20, 22–24, 25) partway down the axis, which served partly to separate the embryo into basal and apical lobes. The basal lobe frequently developed a thick circle of cilia around its lower extremity (Figs. 23–25 —ciliated area shown as wide band).

Exogastrulation had taken place in 15–50 per cent of the bottom forms. Most of these exogastrulae, however, were secondary, in that evagination began only after invagination (as in Fig. 10) had started (Figs. 20–22, 28). All the larvae had an excess of internal cells, and none had developed skeletal elements. A few showed slight bilaterality (Figs. 27–28).

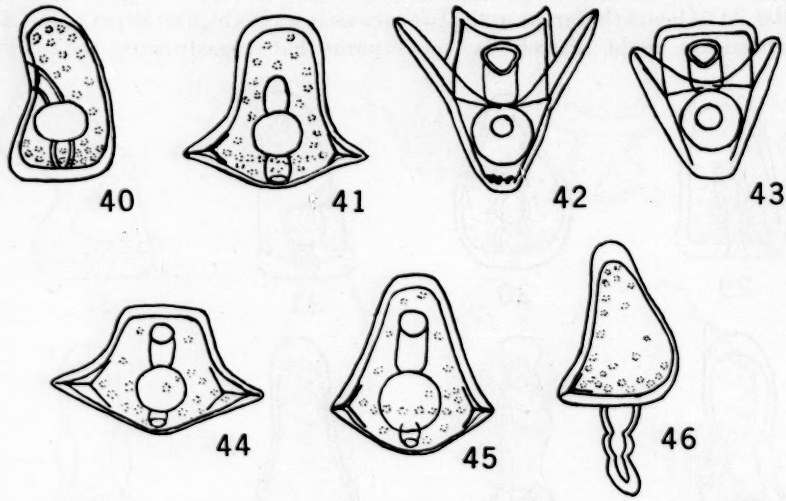
Development in M/800 Na-selenite was not very different from that in M/400. At 24 hours the larvae in the two concentrations could scarcely be told apart. At 48 hours there were also but slight differences. Most of the forms developing in M/800 (Figs. 29–39) were radial, with polar elongation, with ciliated apical knobs, and with an excess of internal cells. The neck region in many was well defined and emphasized by a circle of cilia (Figs. 33, 34, 36). In some, however, the neck was not apparent (Figs. 29, 30, 31). Exogastrulation varied with different lots of eggs, but in some cases it was as high as 80 per cent. Of the forms that exogastrulated, the condition



FIGS. 29–39.—Forty-eight-hour larvae after continuous exposure to M/800 Na-selenite, with treatment beginning at the one-cell stage.

in almost all appeared to be secondary, since these forms had partially invaginated before evagination had begun. The basal ring of cilia was well developed in many (Figs. 31–34, 37–38). Ventrodorsality was noted in only a few, and here it was not distinct (Figs. 31–32, 37). No skeletal spicules appeared at this concentration.

Eggs exposed continuously to M/1,600 near the bottom of the culture, ranged from almost normal plutei down to embryos in which development of skeleton and arms was much inhibited (Figs. 42–46). In general, however, the arms were short and at increased angles, the ventral surfaces were increased in breadth and area, and there was an excess of internal cells. Exogastrulation occurred in about 20 per cent of the embryos. It was com-



FIGS. 40–46.—Figs. 40–41, 48-hour larvae after continuous exposure to M/1,600 Na-selenite, with treatment beginning at the one-cell stage. Figs. 42–46, 48-hour larvae after continuous exposure to M/3,200 Na-selenite, with treatment beginning at the one-cell stage.

Na-selenite were more rapid in development than in higher concentrations. By 48 hours, approximately half had developed ventrodorsality (Figs. 40–41), and, of these, many had developed short anal arms with skeleton. The remainder were elongate radial forms which were comparable to those found when the eggs were treated with M/800. Secondary exogastrulation (to a greater or lesser extent) was found in approximately 40 per cent. Continuous treatment with M/3,200 gave (after 48 hours) almost all bilateral forms, which, while slow-moving and monly, but not always (Fig. 46), secondary.

Aside from its obviously inhibitory action, it seems to be clear from these experiments that the most important effects caused by continuous exposure to Na-selenite are (1) the elongation of the polar axis, (2) the partial or complete elimination of bilateral symmetry, (3) the elimination of skeletal elements, (4) exogastrulation at effective (but not the highest) concentrations, (5) the overdevelopment of a ciliated apical knob, and (6) the development of circles of cilia

about the bases and mid-regions of certain larvae.

II. SIX TO 24 HOURS' EXPOSURE TO NA-SELENITE IMMEDIATELY AFTER FERTILIZATION AND FOLLOWED BY RETURN TO SEA WATER

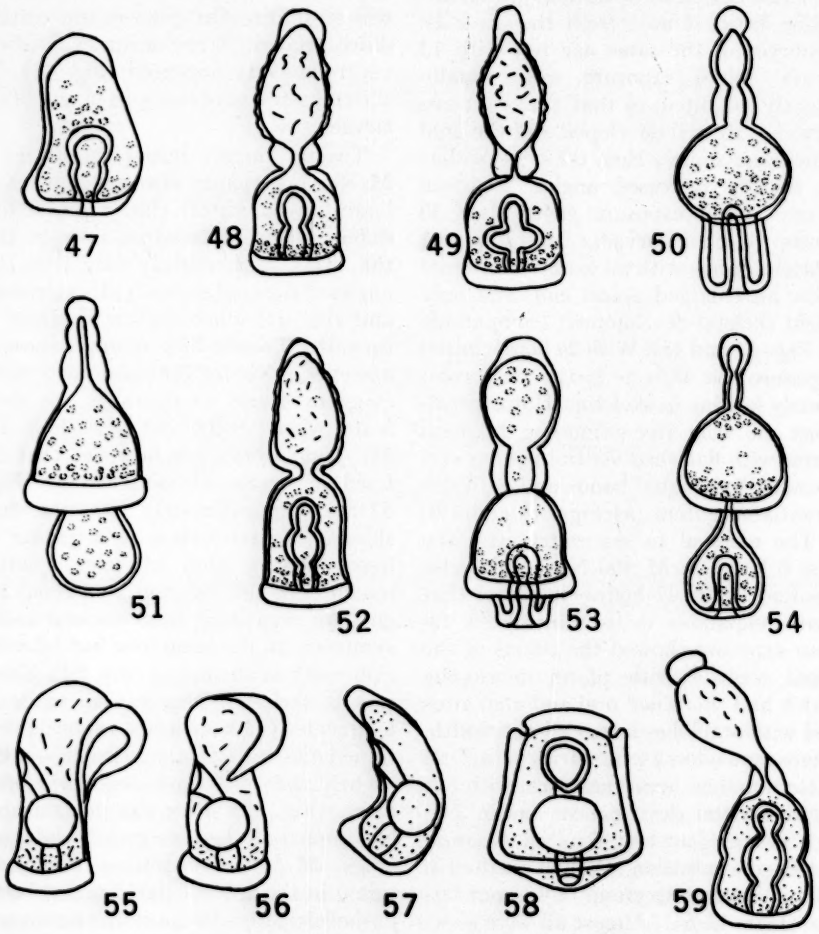
Forty-eight-hour larvae that had been exposed for the first 6 hours of development to M/200 Na-selenite were found on examination to be normal plutei, differing little, if any, from the controls. Embryos of the same age but with 12 hours' initial exposure were usually slightly inhibited, in that the oral arms were not so well developed and the anal arms were shorter than the controls but at slightly increased angles. Eighteen hours' initial exposure gave, after 30 hours (48-hour larvae), free-swimming bilateral forms with an excess of internal cells, an enlarged apical end, and only slight skeletal development (comparable to Figs. 41 and 45). With 24 hours' initial exposure the 48-hour larvae were completely lacking in skeleton. However, almost all were free-swimming bilateral forms with flattened ventral regions surrounded by ciliated bands and with differentiated entera (as Figs. 18 and 19).

The removal to sea water after the first 6 hours in M/400 Na-selenite also resulted (after 42 hours) in plutei that were comparable to the controls. A 12-hour exposure showed the effects of the agent slightly with plutei developing which had shortened oral and anal arms and with oral lobes increased in breadth. There were a few asymmetries in skeletal pattern and an occasional form with but slight skeletal development (as in Fig. 41). Twenty-four hours' initial exposure to this concentration (M/400) resulted in a most interesting group of 48-hour larvae (Figs. 47–51). Almost all were slow-moving bottom forms with an excess of internal cells. Approximately 80 per cent were elongate, radial embryos which were divided into apical and basal lobes (chiefly like Figs. 48 and 49). Most had thickened, ciliated, apical knobs and bands of cilia about the neck and base. Approximately 25 per cent showed exogastrulation (Figs. 50, 51), which in most cases was secondary (Fig. 50). Muscle cells had commonly developed beneath the ectoderm of the apical lobe, which resulted in spasmodic contractions of this structure. The enteron was usually differentiated. A few forms with slight ventrodorsality appeared (Fig. 47). No skeletal elements developed in any of the larvae.

Twelve hours' initial exposure to M/800 Na-selenite also gave (after 36 hours in sea water) chiefly plutei that differed from the controls only in that the arms were slightly inhibited, the angles of the anal arms slightly increased, and the oral lobes slightly enlarged in breadth. Twenty-four hours' exposure, however, gave, for the most part, radial elongate forms comparable to those found when M/400 was used (Figs. 47–51). There were a few, however, that differed in degree of modification (Figs. 52–54). Approximately 50 per cent showed exogastrulation to a greater or lesser degree, and, in many, active muscle cells had differentiated about the gut. An occasional form showed radial symmetry in the basal lobe but bilateral symmetry in the apical (Fig. 52). There was no skeleton. Observations made 24 hours later (72-hour larvae) showed continued differentiation, particularly in the slightly bilateral and less developed forms (Fig. 47). Here the ventral area had commonly become greatly enlarged (Figs. 55–56)—a condition which resulted in the apical ciliated region being pushed dorsally. Also, a stomodaeum and basal ciliated band had usually appeared. In a number of the bilateral forms, however, the ventral region did not enlarge to such an extent, and in these a ciliated

band developed around the entire ventral area (Figs. 57–58). Moreover, most of the larvae did not undergo considerable differentiation between 48 and 72 hours but retained the radial elongate shape characteristic of this treatment (Fig. 59). Also to be noted was the failure of skeleton to differentiate, even though slight muscular contraction could be seen in almost any region.

Removal from M/1,600 Na-selenite after 24 hours' exposure gave over 90 per cent radial (or near-radial), elongate, differentiated forms comparable to those caused by higher concentrations (as Figs. 48, 49, 52). All were near the bottom of



FIGS. 47–59.—Figs. 47–51, 48-hour larvae with the first 24 hours in M/400 Na-selenite and the second 24 hours in sea water. Figs. 52–54, 48-hour larvae with the first 24 hours in M/800 Na-selenite and the second 24 hours in sea water. Figs. 55–59, 72-hour larvae with the first 24 hours in M/800 Na-selenite and the following 48 hours in sea water.

the culture. Ten per cent or less developed into bilateral embryos with flattened or convex ventral areas and with short anal arms containing skeleton (as in Fig. 41).

These data show that an initial 6-hour exposure (during early cleavage) to even the higher concentrations of Na-selenite had little effect and that with 12 hours' exposure the effects were slight. With 24 hours' exposure the picture was very different. In the higher concentrations (M/200) skeleton was inhibited entirely, but practically all the larvae developed bilaterality, flattened ventral areas surrounded by ciliated bands, and differentiated and invaginated entera. With the lower concentrations (M/400–M/1,600) skeleton was inhibited, and most failed to develop bilaterality. Instead, they commonly became much elongated, radial larvae with ciliated bands around constricted necks, active apical tufts, and basal rings of cilia. Exogastrulation occurred in 25–50 per cent.

III. CONTINUOUS EXPOSURE TO NA-SELENITE BEGINNING AT VARIOUS INTERVALS AFTER FERTILIZATION

a) Beginning with the 6-hour blastula stage.—When these stages (Fig. 1) were placed in M/200 Na-selenite, the rate of development was inhibited, but the pattern followed was almost identical with that found when the newly fertilized eggs were exposed to this concentration. That is, after 48 hours (54-hour embryos), approximately 50 per cent were radial, in symmetry with most of these lengthened in the polar direction (Figs. 13–17). The remainder developed into flattened, bilateral forms (Figs. 18–19). All had an excess of internal cells, but skeleton was entirely absent.

Continuous exposure to M/400, beginning with the 6-hour blastula, did not have quite the same effect as when exposure to this concentration began at the one-cell stage. Over half developed into bilateral forms (comparable to Figs. 18–19), while the remainder gave rise to radial (or near-radial) elongate larvae (ranging between those shown in Figs. 15, 29, 30, 35, and 37). Approximately 15 per cent showed exogastrulation, which was usually secondary. Similar treatment with M/800 yielded forms quite like those at M/400. Fifty per cent or more developed bilaterality to a greater or lesser extent (as in Figs. 18, 19, 27, 28), with the remainder being elongate, radial, or near-radial (as Figs. 15, 25, 37) and with a certain amount of polar differentiation. No skeleton developed in either M/400 or M/800. Exogastrulation occurred in less than 25 per cent of those exposed to M/800.

With M/1,600 Na-selenite most of the larvae developed into slow-moving bottom forms which clearly showed the effects of the agent (Figs. 18, 19, 28, 31, 32, 40) in the development of excess internal cells, flattened or convex ventral areas, thickened apical ends, often a basal ciliated band, and the complete absence of skeleton. There were a few (possibly 10 per cent) that developed into the radial elongate forms described for higher concentrations (as Fig. 15). Also, approximately 10 per cent of the total had an evaginated (usually secondary) gut. With M/3,200 the picture was greatly changed. After 48 hours, most were free-swimming plutei (comparable to Fig. 42) with anal arms at increased angles and the oral lobe wider than normal but less differentiated than the controls. There were only a few (as Figs. 15 and 31) which showed the more drastic effects of the agent, and these were confined to the bottom of the culture.

b) Beginning with the 12-hour blastula stage.—This was the period just before (and at the very beginning) of gastrula-

tion (Fig. 2). Primary mesenchyme had appeared, but ventrodorsality could not be seen. The embryos were free-swimming. After 40 hours' exposure to M/200 Na-selenite, the larvae (now 52 hours old) were chiefly slow-moving bottom forms. Bilateral and with greatly flattened ventral areas, they had developed no skeleton (as in Figs. 18 and 19). A ciliated band encircled the ventral side, there was an excess of internal cells, a stomodaeum had appeared, and the enteron was well differentiated. With M/400 and M/800 the picture was not greatly different except that with the lower concentration there was a trace of skeleton in the regions of the anal arms. Also, there were a few in both M/400 and M/800 which, though definitely bilateral, had elongated somewhat in the polar direction and taken on some of the characteristics of larvae where treatment had begun earlier (comparable to Figs. 31 and 40).

c) Beginning with the 24-hour prepluteus stage.—In one experiment 24-hour larvae were exposed to M/800 Na-selenite. When the controls were normal free-swimming 52-hour plutei, the tests were largely cell-filled, inhibited plutei with short anal arms and with oral lobes poorly differentiated (as in Fig. 43).

From these data it was apparent that, when exposure began toward the end of cleavage (6-hour blastulae), more larvae developed bilaterality and fewer showed exogastrulation. Skeletal development, however, still was inhibited as much as when exposure began at the one-cell stage. In general, it can be stated that, except for the highest concentrations, the effects of the agent were somewhat less than when exposure was continuous from the one-cell stage. With treatment beginning at the 12-hour stage, bilaterality developed in practically all forms, and exogastrulation seldom occurred. No skeleton developed in the higher concentrations (M/200), but traces appeared in concentrations (M/400–M/800) that were inhibitory with earlier exposures. With this treatment a few larvae still showed polar elongation. When exposure began at the 24-hour stage, inhibition was more of a general nature (less disproportionate) and resulted chiefly in cell-filled, short-armed plutei.

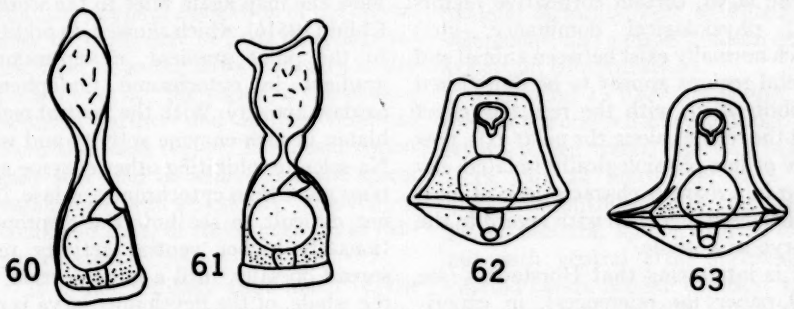
IV. SIX TO 18 HOURS' EXPOSURE TO NA-SELENITE BEGINNING AT THE 6- AND 12-HOUR STAGES AND FOLLOWED BY RETURN TO SEA WATER

Six hours' exposure to M/200 Na-selenite beginning with the 6-hour blastula gave, after 42 hours in sea water (54-hour larvae), chiefly plutei that were quite normal in appearance and only slightly behind the control in degree of differentiation. Eighteen hours' exposure to this concentration and beginning at this stage resulted almost entirely in the development of bilateral forms with no skeleton and flattened ventral regions (as Fig. 18). These forms were to be found both near the top and near the bottom of the cultures.

Six hours' exposure of the 6-hour blastula to M/400 also gave chiefly plutei that were not far different from the controls. However, in approximately 30 per cent the anal arms developed at increased angles and the oral lobes were not well differentiated. Also, an occasional elongate form appeared that reminded one of embryos that had been subjected to more severe treatment with the agent. Eighteen hours' exposure to this concentration and at this stage gave approximately 90 per cent elongate forms that were comparable to those exposed for 24 hours beginning with the one-cell stage (Figs. 47–51). Most were radial, but bilaterality was evident in a few (as in Figs. 28, 47, 52). Exogastrulation (usually secondary) was found in approxi-

mately 10 per cent. No skeleton developed.

Eighteen hours' exposure to M/800, beginning at the 6-hour stage, resulted (30 hours after return to sea water—54-hour larvae) chiefly in bottom forms, of which approximately 20 per cent were radial, elongate embryos (Figs. 60–61). In some of these the apical ciliated region had divided (Fig. 61). The remainder were bilateral flattened larvae with wide ventral areas surrounded by ciliary bands (Figs. 62–63). Skeletal spicules in short anal arms appeared in about 20 per cent of these (Fig. 63). No exogastrulation was seen.



FIGS. 60–63.—Larvae resulting from 18 hours' exposure to M/800 Na-selenite, beginning at the 6-hour blastula stage and followed by 30 hours in sea water.

Twelve hours' exposure to M/200 beginning at the 12-hour stage resulted after 30 hours (54-hour larvae) in the development of bottom- and top-swimming forms that were very much like those exposed continuously to this concentration and beginning at this stage (as Figs. 18, 19). That is, they were almost entirely bilateral forms with flattened ventral areas surrounded by ciliated bands. They possessed a differentiated gut but were completely lacking in skeleton. None of the elongate radial forms was seen. Similar exposure to M/800 also gave forms with flattened ventral areas that were comparable to those treated with M/200. However, with this lower concentration, approximately 50 per cent developed some skeleton in the regions of the anal arms (as in Figs. 44 and 63).

These experiments show clearly that 6 hours' exposure to Na-selenite, beginning at the 6-hour blastula stage, had only slight effects but that 18 hours' exposure (at this stage) to critical concentrations (M/400) gave elongated forms that were chiefly radial and without skeleton. With higher and lower concentrations, the radial forms decreased in number. No radial forms were found in experiments dealing with 12 hours' exposure and beginning at the 12-hour stage, although this treatment gave embryos with flattened ventral areas and, with the higher concentrations, the complete loss of skeletal elements.

DISCUSSION

Child (1941*b*) has shown an apicobasal gradient of what appears to be cytochrome oxidase activity in the eggs of *Dendraster*. This gradient was made apparent in the living eggs by means of the indophenol blue reaction. It was found to be highest at the animal pole. There is evidence that Na-selenite, at certain critical concentrations, may inhibit certain

enzyme activities (succinic dehydrogenase, etc.) to a greater extent than it does those of cytochrome oxidase (University of Wisconsin Biochemists, 1939; Bernheim, 1942). The polar elongation of the larva and the growth and development of the apical lobe following treatment with Na-selenite may well be related to a disproportionate inhibition between apical and more basal regions. In other words, the lower-gradient region is more inhibited, and the apical region grows at its expense. With the elongation of the larva, certain correlative factors (i.e., physiological dominance, etc.) which normally exist between animal and vegetal regions appear to be diminished or obliterated, with the resulting effect that the regions along the polar axis, now more or less physiologically isolated, develop in a manner characteristic of local conditions rather than with regard to the embryo as a whole.

It is interesting that Hörstadius (see 1949 paper for references), in experiments dealing with the mutual influences between animal and vegetal regions in the early sea-urchin embryo, showed that a constriction around the mid-region caused the development of elongate larvae which resembled, in many respects, those caused through the use of Na-selenite reported in this paper. Hörstadius discusses the work of Lindahl (1932, 1936), in which similar phenomena were observed in eggs stretched in the direction of the polar axis in capillary tubes. He repeats Lindahl's explanation that the overdevelopment (disproportionate) of the animal region is because the diffusion of certain substances from the vegetal region is rendered more difficult.

The development of such a large number (as high as 90 per cent in some cases) of radial forms was at first thought to be the result of complete obliteration or flattening-out of the ventrodorsal gradient system through differential inhibition. If this was the situation, then it was difficult to account for the fact that more forms developed bilaterality at the higher concenterations (M/200), while at lower concentrations (M/400–M/1,600) radial forms were more often produced. The development of bilaterality in the higher, but not in the lower, concentrations indicates that a condition of ventralization may have taken place. That is, the ventral area had spread until it surrounded the whole of the larva. In support of this view one may again refer to the work of Child (1941*b*), which showed, in addition to the polar gradient, a ventrodorsal gradient in cytochrome (indophenol) oxidase activity. With the ventral region higher in such enzyme activity and with Na-selenite inhibiting other enzyme systems more than cytochrome oxidase, it is not difficult to see how the disproportionately higher ventral activity may spread dorsally until a large portion, or the whole, of the developing larva is encompassed. A similar situation may exist when maleic acid is used as the test agent (Rulon, 1949). In the high concentrations (M/200), where more bilateral forms appeared, it seems not unlikely that all processes, including those of the ventral region, were strongly inhibited. Such inhibition may well result in a more proportionate (less differential) type of development.

Any interpretation relative to the high percentage of exogastrulae from eggs exposed to Na-selenite must be tentative. It is difficult to understand why the highest concentration (M/200) produced very few, if any, such forms, while lower concentrations (M/200–M/1,600) caused as high as 80 per cent in some instances. As in the case of bilaterality, it appears that the higher concentrations, although more inhibitory, give less in the way of differential (disproportionate) develop-

ment. In earlier work (Rulon, 1950*a*, *b*; 1951) it was pointed out that the inhibition of cytochrome oxidase activity, which appears at the vegetal end shortly before gastrulation (Child, 1941*b*), may inhibit the invagination process and thus cause exogastrulation. This does not seem to be the case with Na-selenite, since this agent is not known for its inhibition of the enzyme. Also, it allows invagination at *higher* concentrations than when evagination is produced. In a preceding paragraph it was pointed out that the physical elongation of the larvae may be important in local morphogenesis. That is, as the larva elongates in the polar direction, the region of dominance becomes farther and farther removed from the basal region. The basal region may now tend to behave as if the apical region had been physically removed—a condition that, of itself, may cause exogastrulation.

It is not unlikely that much of the type of development observed when the eggs and blastulae were exposed continuously to Na-selenite was the result of differential tolerance or conditioning in the sense of Child (see 1949*a*, pp. 197–246). That is, certain regions (apical, ventral), because of greater physiological activity, are better able to tolerate (or condition to) the agent than are regions (basal, dorsal) of lower physiological activity. Indeed, this is what one might expect from an agent that has less effect on enzyme systems (cytochrome oxidase) in which the greatest activity, at the stages considered, is apical and ventral. Initial exposure followed by return to sea water gave evidence of differential recovery (also in the sense of Child, 1941*a*). The enlargement and overdevelopment of regions high in cytochrome oxidase activity, at the expense of those not so endowed, would again be expected with an agent of the type of Na-selenite.

SUMMARY

1. Newly fertilized eggs and early larvae of *D. excentricus* were exposed continuously and for stated intervals to sea-water solutions of M/200–M/3,200 Na-selenite.

2. Over most of the range of concentrations continuous exposure, beginning with the one-cell stage, caused the development of a high percentage of radial, elongated larvae, showing enlarged, ciliated, apical knobs and ciliated bands around mid-regions and basal ends. Skeletal elements were inhibited in development, and exogastrulation occurred at certain concentrations.

3. Six and 12 hours' exposure, beginning at the one-cell stage and followed by return to sea water, had only slight effects, but 24 hours' exposure to the higher concentrations (M/200) gave flattened, bilateral larvae without skeleton but with ventral areas surrounded by ciliated bands. Similar exposure to lower concentrations (M/400–M/1,600) gave chiefly radial, elongated forms with active apical tufts and ciliated bands around constricted necks and basal regions. Exogastrulation was not uncommon.

4. Continuous exposure to the higher concentrations and beginning at the 6- and 12-hour stages gave more forms developing bilaterality and fewer forms showing exogastrulation. Skeleton formation was inhibited. Exposure beginning at the 24-hour prepluteus stage resulted chiefly in cell-filled, short-armed plutei.

5. Twelve hours' exposure of 12-hour larvae to M/200 and M/800 gave flattened, bilateral forms with ventral area surrounded by ciliated bands and with invaginated, differentiated entera. Skeleton formation was completely inhibited in the higher concentrations, but some developed in the lower concentrations.

6. The various modifications are interpreted as resulting, either directly or indirectly, from the greater inhibition of certain enzymes by Na-selenite in the gradient systems of the egg and developing larvae. Differential inhibition, conditioning, and recovery, as well as physiological dominance and isolation, appear as important factors in the interpretation.

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THE MODIFICATION OF DEVELOPMENTAL PATTERNS IN THE SAND DOLLAR BY GLUCOSE[1]

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THE work of Barth and Jaeger (1947) and of Hall and Moog (1948) has indicated that phosphorylations may be of much importance in supplying energy for developmental processes in amphibian eggs. Investigations concerned with the modification of developmental patterns in the sand dollar (Rulon, 1950*a*) have shown that sodium azide is an excellent agent for the production of entodermized exogastrulae. Since azide is an inhibitor of phosphorylations, there is reason to believe that such inhibition may be a factor in exogastrulation and entodermization. However, azide also inhibits other enzyme activities (cytochrome oxidase, catalase, etc.), and such inhibition may be equally or even more important in causing this type of developmental modification.

Since glucose inhibits phosphorylase activity (see Sumner and Somers, 1947, p. 125, for references) and since such activity appears to be important in devel-

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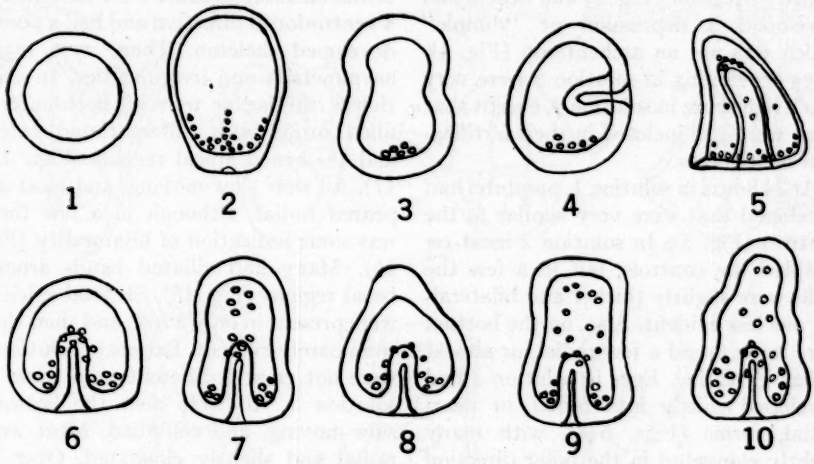
opmental processes, it seemed in line with our general research program (Rulon, 1948, 1949, 1950*a*, *b*, 1951, 1952) to test the effects of this agent on the developing egg of the sand dollar.

MATERIAL AND METHODS

The sand dollars, *Dendraster excentricus*, were collected by dredging from Monterey Bay during the breeding season (June–August) and maintained in running sea water in the aquarium room at the Hopkins Marine Station. Eggs were taken from ripe females, washed several times, and fertilized by the addition of a few drops of sperm suspension. Fertilization, as evidenced by the appearance of fertilization membranes, was high. The eggs were then placed in mixtures of sea water and sugar solution. All cultures were kept in covered finger bowls at a temperature of 18° ± 1° C. Crowding in cultures was prevented. No smoking was permitted in the laboratory. Controls in sea water were always run alongside the tests. Observations were made of the developing embryos at frequent intervals.

The test solutions used in the following experiments were:

1...100 cc. sea water + 20 cc. 1.00 M glucose[2]
2...100 cc. sea water + 40 cc. 1.00 M glucose
3...100 cc. sea water + 60 cc. 1.00 M glucose
4...100 cc. sea water + 80 cc. 1.00 M glucose
5...100 cc. sea water + 100 cc. 1.00 M glucose



FIGS. 1–10.—Fig. 1, normal 6-hour blastula. Fig. 2, normal 12-hour early gastrula. Figs. 3–4, 12-hour larvae that have been exposed continuously to strong solutions (sols. 4 and 5) of glucose. Fig. 5, normal 24-hour prepluteus. Figs. 6–8, 24-hour larvae that have been exposed continuously to moderate solutions (sol. 3) of glucose. Fig. 9, 24-hour larva that has been exposed continuously to solution 4. Fig. 10, 24-hour larva that has been exposed continuously to solution 5.

EXPERIMENTAL

I. CONTINUOUS EXPOSURE TO GLUCOSE BEGINNING AT THE ONE-CELL STAGE

After 6 hours' exposure to solution 1, blastulae had developed that were very similar to the control (Fig. 1). With solutions 2 and 3 the individual cells tended to be larger and less transparent. With

[2] Dextrose—Anhydrous (C.P.), Baker and Adams. The 1.00 molar solution was made up with distilled water. This concentration of sugar was approximately isosmotic with the protoplasm of the uncleaved egg of *Dendraster*.

solutions 4 and 5 the early blastulae had still fewer, larger, and more granular cells. After 12 hours' exposure the eggs in solutions 1 and 2 had reached the late blastula stage and, except for slightly thicker walls, could not be distinguished from the controls (Fig. 2). In solution 3 the larvae were free-swimming, but there was definitely less mesenchyme than in the controls. With solution 4 many were like those in solution 3, but some were slightly irregular (Fig. 3) and others had developed a depression or "dimple" which was not an archenteron (Fig. 4). Eggs developing in solution 5 were very much like those in solution 4, except that some were still inclosed in their fertilization membranes.

At 24 hours in solution 1, preplutei had developed that were very similar to the controls (Fig. 5). In solution 2 most resembled the controls, but in a few the walls were slightly thicker and bilaterality was less evident. Also, on the bottom were to be found a few radial, or almost radial, gastrulae. Eggs in solution 3 had developed chiefly into radial, or near-radial, forms (Figs. 6–8), with many slightly elongated in the polar direction (Fig. 7). Some, however, still had slight bilaterality, and a few were slightly irregular. In all the embryos the walls were definitely thicker than in the controls. In solution 4 the eggs had developed chiefly into free-swimming, slightly elongated, radial forms with a slight excess of mesenchyme (Fig. 9). In most, the walls were unwrinkled and the enteron slightly inhibited. In solution 5 the embryos were chiefly free-swimming, irregular, wrinkled, slightly elongated forms (Fig. 10). Many had a slight excess of internal cells, and there were a few grossly inhibited and cytolyzing forms at the bottom of the culture.

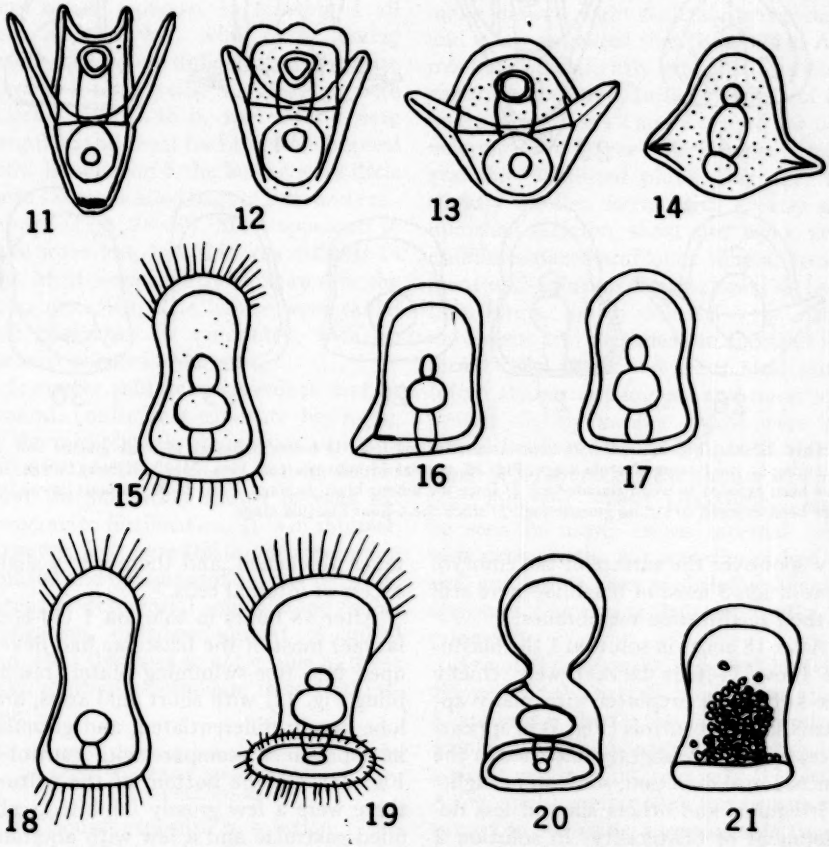
At 48 hours the eggs in solution 1 had developed into plutei that presented a slightly granular appearance and contained oral lobes that were slightly less well developed than those of the controls (Fig. 11). In solution 2, the larvae showed considerable inhibition, ranging from free-swimming granular plutei with poorly developed oral lobes and short anal arms (at various angles) down to grossly inhibited bottom forms (Figs. 12–14). Many were so granular in appearance that it was difficult to see internal structures. Most were flattened in a ventrodorsal direction and had a poorly developed skeleton. There were many asymmetries and irregularities. In solution 3, the larvae were all bottom, cell-filled forms with differentiated entera and thickened apical regions (Figs. 15–17). All were slow-moving, and most appeared radial, although in a few there was some indication of bilaterality (Fig. 16). Many had ciliated bands around basal regions (Fig. 15). Skeletal spicules were present in only a few, and then they were barely evident. Larvae in solution 4 were not greatly different from those in solution 3. All were near the bottom, slow-moving, and cell-filled. Most were radial and slightly elongated. Over 50 per cent had the basal ciliated band (Fig. 15). No skeleton was seen. Those in solution 5 had developed into slow-moving, radial, cell-filled, bottom forms which usually had a ciliated band around the basal region (Figs. 18–21). All had a thickened apical region, and none had skeleton. Many had become elongated, with a constriction appearing about midway along the polar axis which separated (at least in part) the embryo into apical and basal lobes. In approximately 50 per cent the gut had broken down into its component cells (Fig. 21).

These data show that continuous exposure following fertilization to the higher concentrations of glucose has certain definite effects. Cell division is re-

tarded and "wrinkling" or "dimpling" is caused in the late blastular stages. In later development the larvae tend (1) to develop into radial forms, elongated in the polar direction, (2) to show failure in skeletal development, (3) to develop a thickened, ciliated, apical end and a basal ciliated band, (4) to show breakdown of gut into component cells, and (5) to develop a constriction at about midway along the polar axis of the elongate forms.

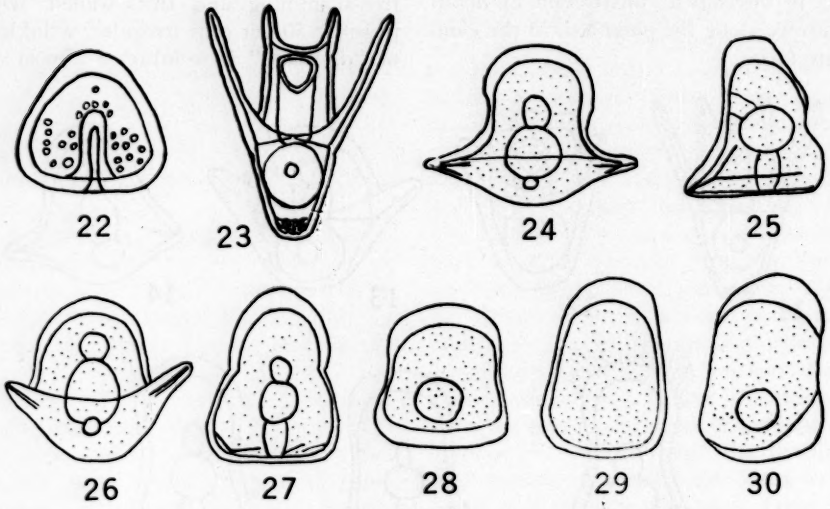
II. CONTINUOUS EXPOSURE TO GLUCOSE BEGINNING WITH THE MID-BLASTULA

When 6-hour blastulae had been exposed to solution 1 for 6 hours, most had developed into free-swimming, thick-walled, slightly irregular forms. Approximately 20 per cent were distinctly wrinkled or contained "dimples" (as in Figs. 3 and 4). In solution 2 most were free-swimming and thick-walled, with possibly 50 per cent irregular, wrinkled, or "dimpled." In solution 3 almost all



FIGS. 11–21.—Fig. 11, normal 48-hour pluteus. Figs. 12–14, 48-hour larvae that have been exposed continuously to weak solutions (sol. 2) of glucose. Figs. 15–17, 48-hour larvae that have been exposed continuously to moderate solutions (sol. 3) of glucose. Figs. 18–21, 48-hour larvae that have been exposed continuously to strong solutions (sol. 5) of glucose.

were near the bottom of the culture and, although showing considerable ciliary activity, were thick-walled, wrinkled and/or with "dimples." In solutions 4 and 5 almost all were slow-moving bottom forms which were wrinkled and irregular and usually showed one or more deep "dimples" which resemble archentera (as in Fig. 4) but were to be found at almost any spot over the surface of the embryo. In solution 5 most of blastulae were still in their fertilization membranes.



FIGS. 22–30.—Fig. 22, radial 24-hour larva resulting from 18 hours' exposure to weak glucose (sol. 2), beginning at the 6-hour blastula stage. Fig. 23, normal 54-hour pluteus. Figs. 24–27, 54-hour larvae that have been exposed to weak glucose (sol. 2) since the 6-hour blastula stage. Figs. 28-30, 54-hour larvae that have been exposed to strong glucose (sol. 5) since the 6-hour blastula stage.

After 18 hours in solution 1 the blastulae (now 24-hour larvae) were chiefly free-swimming preplutei with many approaching the controls (Fig. 5) in appearance. A few were slightly flattened in the ventrodorsal direction, some were slightly irregular, and others showed less development of bilaterality. In solution 2 the larvae were free-swimming, but the walls were thicker than in the controls. Most were radial or near-radial in symmetry (Fig. 22), and slight irregularities were common. In solution 3 most were extensively wrinkled and irregular, although a few were comparable to those in solution 2 (Fig. 22). With solutions 4 and 5 the wrinkled condition had increased, although the radial pattern was usually evident. Also, with these higher concentrations the gastrular walls remained much thickened, and there was a slight excess of internal cells.

After 48 hours in solution 1 (54-hour larvae) most of the blastulae had developed into free-swimming plutei (resembling Fig. 12) with short anal arms, oral lobe poorly differentiated, and granular in appearance (compare with control—Fig. 23). On the bottom of the culture there were a few grossly inhibited, cell-filled gastrulae and a few with aberrant angles to the anal arms, duplications of anal arms, etc. In solution 2 most were cell-filled bottom plutei (Figs. 24–26)

with undifferentiated oral lobes and with short anal arms which were usually aberrant and askew. Most were bilateral and flattened ventrally, but others were radial in symmetry (Fig. 27). Skeleton, while present, was much inhibited. In solution 3 the larvae had developed into slow-moving, cell-filled bottom forms, usually with slight bilaterality and a slight amount of skeleton. The apical end was usually thickened, and the gut had differentiated. Most of the forms showed considerable irregularity in shape and were almost opaque. In solution 4 all were slow-moving, with most having entera which were difficult to see because of many internal cells. In only a few were skeletal spicules to be seen. Many were irregular, and most had thickened apical ends. In solution 5 the larvae were little more than cell-filled bags of cells and cell-clumps (Figs. 28–30). Most appeared to have some gut, but this was difficult to say. Most were slightly elongated in the polar direction. The larvae were radial and misshapen in symmetry, with no skeletal spicules to be seen.

It may be said from these data that, in general, continuous exposure beginning at the mid-blastula stage gave a picture of inhibition that differed only in degree from the inhibition shown in eggs exposed since fertilization. It is of interest, however, that here the larvae were more stunted and opaque and did not show the extent of differential conditioning (polar elongation, overdevelopment of apical end, etc.) that was so noticeable in those with continuous exposure from fertilization.

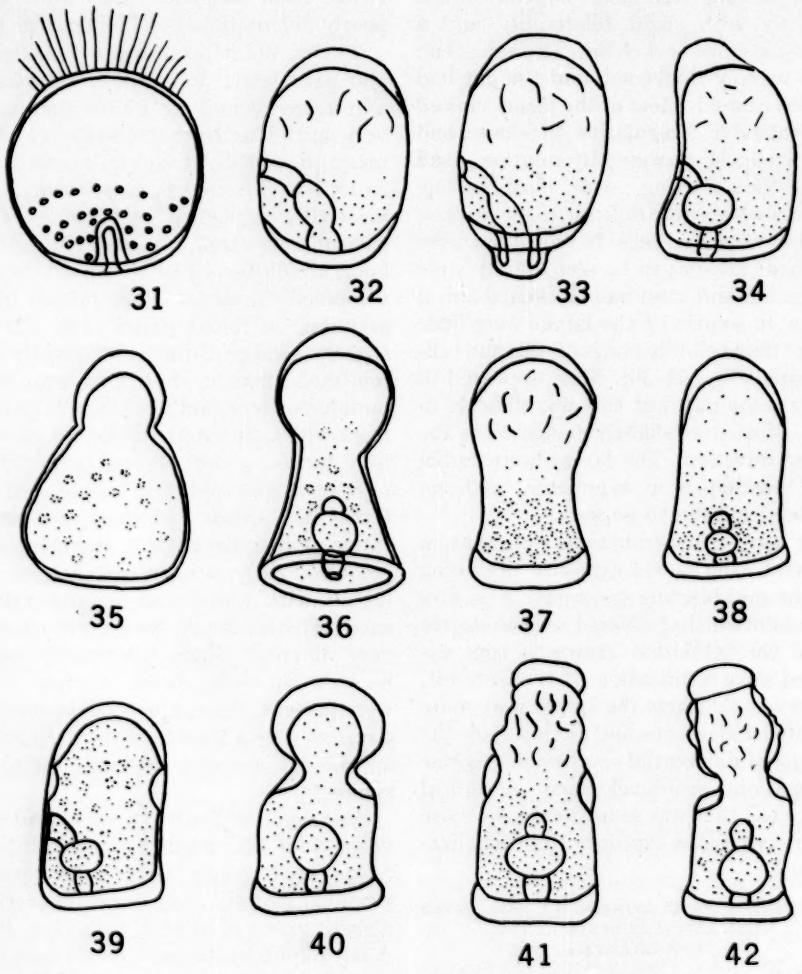
III. RETURN FROM GLUCOSE TO SEA WATER WHEN EXPOSURE BEGAN AT THE ONE-CELL STAGE

Six hours' exposure to solution 1, followed by 42 hours in sea water, resulted chiefly in normal-appearing plutei, which, in many cases, seemed to be slightly more advanced than the controls. There were a few, however, that showed extra anal arms and slightly aberrant skeleton. Similar treatment with solutions 2–5 gave, after 42 hours, a few free-swimming plutei which approached the controls (Fig. 1), but most were slightly granular forms with shortened anal arms and poorly differentiated oral lobes (Fig. 12).

Twelve hours' exposure to solution 1 also gave (after 36 hours in sea water) a few free-swimming plutei that were very much like the controls (Fig. 11), but many showed slight skeletal aberrancies, and a few appeared slightly swollen. Almost all were slightly granular. No exogastrulae appeared. Initial exposure of 12 hours to solutions 2 and 3 caused the development of larvae which ranged from granular, inhibited plutei (Fig. 12) to slightly swollen forms with greatly diminished skeleton, short anal arms, and undifferentiated oral lobes. Similar treatment with solution 4 gave almost all bottom forms, which showed very little movement and contained an excess of internal cells. Most had short anal arms (often at aberrant angles), and many appeared slightly swollen. Most were bilateral with apical end slightly thickened. With solution 5 the picture was not very different. Slight bilaterality could be seen in most, excess internal cells were present, the gut was diminished in size, and only a trace of skeleton (if any) appeared. Some were definitely radial in symmetry.

As noted previously, eggs that had developed for the first 24 hours in solution 5 appeared chiefly as free-swimming, wrinkled, slightly elongate embryos with a slight excess of internal cells (Fig. 10). A few minutes after such forms were returned to sea water a remarkable transformation had taken place. Most of the larvae had become swollen to over twice their normal size. They were now top-

swimming, thin-walled spheres with slightly thickened apical and basal regions and with apical ciliated tufts greatly extended (Fig. 31). The entera had not increased in size. A few did not swell. At 48 hours (24 hours after return to sea water) these larvae had developed into elongate, swollen, thin-walled, slow-moving, bottom forms (Figs. 32–36). Apical and basal thickenings were usually present, and there were usually small differentiated entera. In a few (5 per cent), basal ciliated bands were particularly distinct (Fig. 36). Twenty-four hours



FIGS. 31–42.—Fig. 31, swollen larva, as seen a few minutes following transfer to sea water, after 24 hours' exposure to strong glucose (sol. 5). Figs. 32–36, 48-hour larvae with the first 24 hours in strong glucose (sol. 5) and the second 24 hours in sea water. Figs. 37–42, 72-hour larvae with the first 24 hours in strong glucose (sol. 5) and the following 48 hours in sea water.

later many of these swollen, thin-walled forms had undergone a certain amount of differentiation (Figs. 37–42). All had thickened apical and basal regions. In many, constrictions (necks) had formed to separate to a greater or lesser degree apical from basal lobes. In a few (5–10 per cent) ciliated bands had developed in the region of these constrictions (Figs. 40–42). Entera were present in most, and muscular action could commonly be detected near apical regions. Only a few gave any evidence of bilaterality, and these only in the position of the stomodaeum (Fig. 39).

From these experiments it was concluded that 6 hours' initial exposure to low concentrations of glucose had little or no effect on the developing egg of *Dendraster* and that with the higher concentrations the effects were slight. With 12 hours' exposure to the low concentrations the effects were small, but the same period of exposure to the highest concentrations gave, in general, larvae that were slightly swollen, with only slight bilaterality, an excess of internal cells, small entera, and skeleton greatly diminished or absent. Osmotic effects were clearly demonstrated following the return to sea water of larvae that had been exposed for the first 24 hours to the highest concentration of sugar. Such larvae became greatly swollen through entrance of water into the blastocoel. With time, most of these swollen larvae developed into elongated, radial forms with a certain degree of differentiation (i.e., thickened apical and basal regions, constricted mid-region).

IV. THE RETURN FROM GLUCOSE TO SEA WATER WHEN EXPOSURE BEGAN AT THE 6-HOUR BLASTULA STAGE

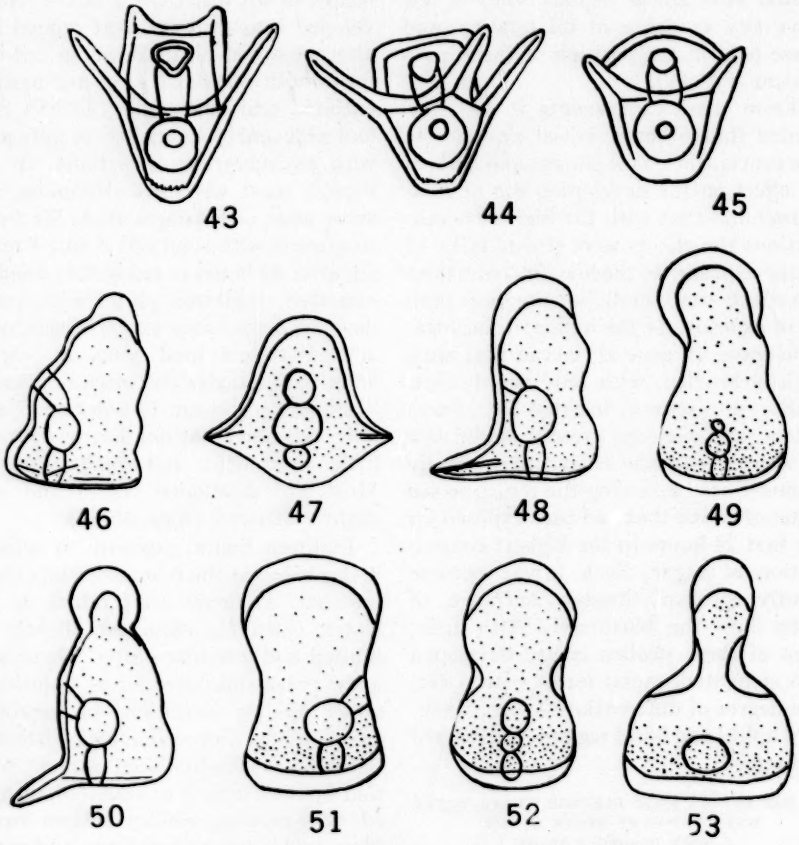
Blastulae were placed in the five different solutions of glucose for 6- and 18-hour periods, after which they were returned to sea water.

Six hours' exposure to solution 1 had only slight effects. When taken from the test solution, approximately 20 per cent of the blastulae were "dimpled" or distinctly wrinkled. However, after 42 hours in sea water, recovery was almost complete and normal-appearing plutei had developed. As noted previously, 6 hours' exposure of blastulae to solution 2 gave approximately 50 per cent wrinkled or dimpled forms. Forty-two hours after return to sea water these larvae had developed into embryos that ranged from almost normal plutei down to cell-filled forms with short anal arms and undifferentiated oral lobes (Figs. 43–45). Some had wide oral lobes, and there were many with asymmetries, distortions, etc. Although most were free-swimming, anal arms were often duplicated. Six hours' treatment with solutions 3 and 4 resulted, after 42 hours in sea water, chiefly in distorted, inhibited plutei with poorly developed oral lobes, excess mesenchyme, and shortened anal arms at various lengths and angles and often duplicated. Six hours' exposure to solution 5 gave only cell-filled bottom forms with but little movement and slight skeleton. Most had developed entera and were slightly bilateral (Figs. 46–47).

Eighteen hours' exposure to solution 2, beginning at the 6-hour blastula stage, resulted, 30 hours after return to sea water, chiefly in distorted, slightly inhibited and granular plutei. There were a few grossly inhibited forms. Solution 3, under similar conditions of exposure, gave chiefly free-swimming, distorted, cell-filled, much-inhibited plutei. Solution 4 gave a variety of inhibited, distorted, slow-moving, swollen bottom forms. Most had a trace of skeleton and many internal cells. Most had entera as well as enlarged (though undifferentiated) apical ends (Figs. 48–50). Solution 5 gave swollen, slow-moving, bottom forms, elongat-

ed in the polar direction. Most had entera, but in most (not all) skeletal spicules were absent. Most were of slightly irregular radial symmetry, but some were slightly flattened, apparently in a ventrodorsal direction. Many were not very different from those caused by an initial 24-hour exposure to the higher concentrations (Figs. 51–53).

These experiments showed that the 6-hour blastula was more affected by 6 hours' exposure to the various concentrations of glucose than was the newly fertilized egg. Certain of these effects may have been related to osmotic factors, but direct action of the agent on developmental processes was also evident. Eighteen hours' exposure, beginning at the 6-hour stage, gave a variety of forms, depending upon the concentration of su-



FIGS. 43–53.—Figs. 43–45, 54-hour larvae with 6 hours' exposure to weak glucose (sol. 2), beginning at the 6-hour blastula stage. Figs. 46–47, 54-hour larvae with 6-hours' exposure to strong glucose (sol. 5), beginning at the 6-hour blastula stage. Figs. 48–50, 54-hour larvae with 18 hours' exposure to solution 4, beginning at the 6-hour blastula stage. Figs. 51–53, 54-hour larvae with 18 hours' exposure to solution 5, beginning at the 6-hour blastula stage.

gar. With the highest concentrations, elongated, almost radial forms, deficient in skeleton, were obtained.

DISCUSSION

To this author, at least, the effects of glucose on developmental processes have been surprising as well as extremely interesting. In general, glucose in solution has not been thought of as inhibitory to the embryo but instead as furnishing energy or, at the very least, as being inert. Spratt (1949) has shown that early chick embryos taken from the yolk survive for a time and undergo morphogenesis and differentiation on a mixture of glucose and Ringer's solution. In the present experiments, however, high concentrations of glucose were definitely shown to inhibit cleavage, skeletal formation, and general rate of development. Differential effects arising from differential inhibition, conditioning, and recovery were also found.

Continuous exposure to the higher concentrations of glucose gave a picture of inhibition with differential conditioning of certain regions. The larvae failed to develop bilaterality and skeleton. The gut, though reduced in size, invaginated and differentiated except at the highest concentration, where there was a tendency toward dissociation of the entodermal cells. The embryo tended to elongate in the polar direction, with the apical end becoming greatly enlarged and ciliated and with a circle of cilia developing about the basal region. In many, a neck appeared which tended to divide the larva into apical and basal lobes.

Any explanation of these profound effects on morphogenesis and differentiation is not easy and must be tentative. There are certain factors, however, that seem important and may offer at least a partial interpretation. Child (1941) showed a polar gradient of indophenol oxidase (cytochrome oxidase) activity in the eggs and early developmental stages of this species. The highest activity was at the animal pole. The present experiments indicate that glucose inhibits less the activity of the apical region and thereby permits it to elongate and enlarge (but not to differentiate as oral lobe) at the expense of more basal regions. With the elongation of the larva, correlative factors which normally exist between animal and vegetal regions may be diminished to a certain extent to permit a degree of local differentiation such as a basal circle of cilia and a neck.

The action of glucose on developmental pattern is complicated by the presence of osmotic factors. Although the solutions used were apparently isosmotic with the egg and cells of the embryo, it has been found (Moore and Burt, 1939; Moore, 1940) that the blastular walls of *Dendraster* become relatively impermeable to sugar after the tenth segmentation, although they remain freely permeable to the salts of sea water. The appearance of "dimples" and irregularities in the last blastula may be explained on this basis. Normally, the blastocoel enlarges in the late blastula stage, but the increase in volume can be only by the entrance of sea water. With embryos in a sugar solution from the beginning of cleavage, the sugar concentration of the blastocoel fluid is equivalent to that of the external medium. Water entering would reduce the concentration of sugar in the fluid below that of the outside solution, which would result in water being osmotically lost from the blastocoel. Thus the volume of the blastocoel tends to remain constant despite the increase in area of the blastular walls.

The transfer from sugar solutions to sea water and from sea water to sugar solutions in the later stages of development leads to swelling or shrinking. The

most pronounced effects of osmotic factors on morphogenesis were found when eggs, that had developed for the first 24 hours in strong sugar solution (solution 5), were transferred back to sea water. Within a few minutes these embryos had become swollen to over twice their normal size by the entrance of water into the blastocoel. This caused a tremendous stretching (with cell displacement) of blastular walls (Fig. 31). When these larvae differentiated it was evident that there had been a disruption of the mutual influences which normally exist between animal and vegetal regions. In other words, the swelling, as well as the direct action of the sugar (presumably on such chemical processes as phosphorylation, etc.), tended to cause the various regions to differentiate semi-independently. In 5–10 per cent of the larvae this condition went far enough to produce a radial, elongated type with ciliated apical knob and base as well as a neck with a ciliated band (Figs. 41–42). This type also showed the development of a reduced, but differentiated, gut and the presence of functional muscle cells but the entire absence of skeleton.

Certain experiments on the effects of Na-selenite on developing eggs of this form (Rulon, 1952) have given elongated larvae that were not very different from those found in the present experiments with glucose. As in the case of glucose, an interpretation, based on the elongation of the larva with loss of correlative factors, was given. This does not mean that the effects of Na-selenite and glucose are similar in all respects. There are also many differences. Not only is Na-selenite a much more effective agent in causing the elongated differentiated forms, but under certain conditions approximately 80 per cent of the larvae undergo exogastrulation. The only explanation to be suggested at this time is that Na-selenite is able to effect a more complete physiological isolation of the vegetal from the animal regions. This causes the vegetal region to behave as if it were mechanically severed from the animal region. Such a condition, itself, may lead to exogastrulation and entodermization. It is also of interest to mention here that Hörstadius, through the use of a silk fiber constriction, and Lindahl (see Hörstadius, 1949, for references), through the stretching of eggs in a capillary tube in the direction of the egg axis, have induced sea-urchin embryos to develop into elongated and animalized forms. These authors believe that such animalization appears because of a certain degree of inhibition of diffusible substances from the vegetal region.

Although both glucose and azide are known to inhibit phosphorylations, their actions on developmental pattern are quite different. Under certain conditions (see Rulon, 1950) azide grossly inhibits apical development while promoting exogastrulation and entodermization. Glucose has opposite effects in causing an increase in size of the animal region with a decrease in size of entoderm. Also, in the case of glucose, exogastrulation occurs with no more frequency than it does under control conditions. These data may indicate that the inhibition of phosphorylation is not directly responsible for exogastrulation and entodermization.

In the normal development of bilaterality in *Dendraster* Child (1941) has found a gradient of indophenol oxidase activity decreasing from the ventral to dorsal regions and preceding any morphological change in this direction. The obvious interpretation, at this time, is that strong inhibition by glucose prevents the development of the ventrodorsal gradient system and thereby permits the embryo to develop along the lines of a radial symmetry. However, work with

maleic acid and Na-selenite (Rulon, 1949, 1952) has indicated that under certain conditions a "ventralization" may take place. That is, agents which exert less inhibition on ventral activity than on dorsal activity may permit the ventral field to spread until it surrounds the egg. Since there is nothing in the present work which indicates that this is not the case in the development of radial forms in glucose, no definite interpretation can be given at this time.

SUMMARY

1. Newly fertilized eggs and 6-hour blastulae of *Dendraster* were subjected to a range of glucose solutions (100 cc. sea water + 20–100 cc. glucose) for varying intervals of time.

2. Continuous exposure to the higher concentrations retarded cell division and caused a wrinkling in the early larvae. As development proceeded, radial forms, elongated in the polar direction, with enlarged apical tufts and with basal ciliated bands, appeared. Skeletal elements failed to form, and the entoderm tended to break down into its component cells. Exposure, beginning at the blastula stage, showed less in the way of differential conditioning than when exposure was continuous from fertilization.

3. Larvae, developing for the first 24 hours in the higher concentrations, became greatly swollen (through osmotic action) when returned to sea water. These enlarged forms gave rise to thin-walled embryos with thickened apical and basal regions. In many, a constriction developed which partially separated apical from basal regions. Skeleton did not form, but muscle cells commonly differentiated. Most were radial in symmetry. Eighteen hours' treatment (between the 6- and 24-hour stages) with the same concentrations was slightly less effective.

4. Interpretation of the effects of strong glucose on morphogenesis in the sand dollar is based on a differential action of the agent on the gradient systems of the developing embryo. This, together with osmotic effects, may diminish certain correlative factors normally existing between animal and vegetal regions and thereby permit a degree of local differentiation.

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THE PROTEIN METABOLISM OF SUNFISHES OF DIFFERENT AGES[1]

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INTRODUCTION

THE nutrition of fishes has intrinsic biological interest because of the phylogenetic position which fishes occupy among the vertebrates. The subject may also have interest for aquatic ecologists who are concerned with the problem of production as defined by Clarke (1946) and elaborated with regard to fishes by Ricker (1946) and Ricker and Foerster (1948). It is from this standpoint that the writer has initiated the present experiments. Production rate is defined as the amount of organisms formed within a body of water per unit time. Therefore, growth is one of the most important considerations involved in this concept. Growth, in turn, is dependent upon an adequate food supply. Fish usually have a natural food supply sufficient to insure growth, and it would be desirable to learn the efficiency with which these animals are able to utilize food for growth. Although the ecologist is most interested in events occurring in nature, it is necessary that he turn to laboratory experimentation for determinations of this kind.

Field studies of food consumption by fishes are limited to a description of the species composition and quantity of foods found in fish stomachs. A voluminous literature has accumulated about this subject. Although this work has been necessary to learn what foods are available to the fish, the important question of how the food is converted into the product of main interest—fish—remains unanswered by this approach. This report is intended to describe some information, obtained by feeding experiments in the laboratory, regarding the efficiency of protein utilization by two species of sunfish. It is hoped that studies of this kind will prove to be valuable in determining some aspects of fish production.

A considerable quantity of material is available concerning the efficiency of food conversion by small fish, particularly trout, in hatcheries. The main aim of these experiments has been to get an economical food which will produce rapid growth. The gross measurement of live weight has usually been employed to measure differences in quality between foods. Water and fat content of the fish influence live weight a great deal, and this might account for the large variations in efficiencies reported. At best, results based on live weight can be only rough indicators of the efficiency of food conversion, and they will not be fully reviewed here. Pentelow (1939), Dawes (1931), and Moore (1941) have contributed experimental material about the efficiency of food conversion in the brown trout, plaice, and sunfish by using live

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Much of the success of these experiments is due to the careful work of Mr. Paul Alexander, who acted as my laboratory assistant.

weights, and their data show great variations. Dawes concluded that the variation was so great that it was not possible to postulate even an approximately constant index of efficiency for any short interval during the growth period. The author felt that more exact nutritional methods would possibly yield better results. Morgulis (1919) used such procedures, but his experiments never reached fruition. Karsinkin (1939) and Ivlev (1939*a*, *b*, *c;* 1945) have obtained valuable information by detailed analysis of food and fish involved in feeding experiments. A group of workers at the Cortland Fish Hatchery in New York (1943–49) has accumulated a great deal of material on the nutrition of fishes.

The influence of age on the efficiency of food conversion by fish is the object of the present work, because this viewpoint provides a more complete understanding of the food-chain relationships between the fish and its supply of food. Specifically, the protein metabolism by fish of different ages has been emphasized, since proteins, in general, are the most active organic materials in living organisms. Lipids, carbohydrates, water, vitamins, and mineral matter are also essential for growth, but they may be regarded as fluctuating about a quantity of protein during the course of life. Ivlev (1945) has stimulated work in this direction by his consideration of the problem from the point of view of the transfer of energy from one part of the food chain to another. He reports that young fish utilize their available energy more efficiently than do older fish. Ivlev's efficiency of energy transfer from food to fish is not equivalent to our efficiency of protein transfer, although both are probably intimately associated and perhaps both are of the same order of magnitude.

This study was particularly designed to investigate gross effects. Although the influence of temperature and quality of food are important considerations, it was felt that the main lines of attack had to be drawn first. The capacity of the laboratory was not sufficient to handle large numbers of fish and the consequent analyses which would be required, but the results seem significant enough to report.

DESIGN OF THE EXPERIMENT

The main objective was to feed fish of different ages sufficient food to insure growth. The retention of protein by the fish during the investigation could be judged only by knowing the composition of the experimental fish at the beginning of the experiment. Therefore, a sample fish was selected to represent each of the experimental fish when feeding commenced. The sample fish were approximately the same size as the experimental fish. They were collected at the same time and were treated in exactly the same way as the experimental fish until the moment when the latter were introduced into aquaria. After being brought into the laboratory, all fish were fasted for 3 days in order to empty their alimentary tracts. The sample fish were subjected to the following measurements: wet weight, dry weight, and nitrogen content.

The experimental fish were killed at the end of a 50-day feeding period, and they were analyzed in the same way as the sample fish. The nitrogen content of the experimental fish at the beginning of the experiment was estimated from the sample fish analysis, and the actual nitrogen content of the experimental fish at the termination of feeding was determined. The difference between the nitrogen content of the fish at the beginning of the experiment and the nitrogen content at the end represented the nitrogen retained by the fish. This procedure is similar to one described by McCollum, Orent-Keiles, and Day (1939). The pro-

cedure has usually been used to test the growth of rats fed different foodstuffs.

Appropriate measurements of the food and feces were made to determine the efficiency of protein absorption by the fish.

MATERIALS

FISHES

The longear sunfish (*Lepomis megalotis megalotis*) and green sunfish (*Lepomis cyanellus*) were chosen for study because they are hardy and adapt quickly to aquarium life. Activity in the room did not disturb them after a short time, and they remained quietly at rest in the corners of the aquaria. Both species are members of the teleost family Centrarchidae, which is one of the most abundant families of fishes found in midwestern lakes and streams. This family is of considerable interest economically, because it is subject to heavy sport-fishing pressure. It was also particularly advantageous to use centrarchid fish because the growth pattern of the group is well known from numerous scale studies.

Both species were obtained from creeks near Bloomington, Indiana. Selection of fish in the field was made on the basis of length, as an indicator of age. Several fish of each size were brought into the laboratory. Differences in length and weight were considered to be an indication of a difference in physiologic age of the fish, since individuals of the same species have about the same growth potential. A discussion of physiologic age is included below.

FOOD

Live food was used to feed the fish, principally because nutrient material is not dissolved when the food is introduced into the water. Larvae of *Tenebrio molitor*, usually called "meal worms," were selected as food because they can be cultured in large quantities in the laboratory and because they simulate the natural food of the fish to some degree. Insect larvae are one of the main constituents of the diet of these sunfishes in nature. The larvae were grown in large metal tanks, where they were fed commercial chick mash. The larvae were stored in a refrigerator; the cool environment inhibited growth and prevented pupation.

In general, smaller meal worms were fed to the smaller fish and larger ones to large fish. There was very little difference in the nitrogen content of small and large meal worms: dried samples of large meal worms contained 9.72 per cent nitrogen, and small meal worms contained 9.45 per cent. An average value of 9.58 per cent nitrogen has been used for total nitrogen calculations.

METHODS

FEEDING

The experiments were performed in aquaria of 50- and 19-liter capacity. Small fish of 10 g. or less were kept in the small tanks, and the rest occupied the large tanks. The aquaria were filled with tap water, which was aerated during the entire experiment.

Each fish was fed a daily ration of meal worms which were weighed on a triple-beam balance having a sensitivity of 0.05 g. The fish were begun on a diet of 3 per cent of their body weight per day and were fed reduced amounts only as they refused to eat all the worms offered. Usually the fish began to refuse food after 20 or 30 days, and from that time on feeding was *ad libitum*. Worms that were not consumed were removed from the aquaria, dried on absorbent paper, weighed, and subtracted from the total amount offered.

The fish were changed to clean aquaria after a period of 10 days. The water in the new aquaria was aerated for at least

24 hours prior to the change, in order to remove chlorine and to allow the water to reach room temperature. At the time the fish were changed, each was weighed to within 0.1 g. after the superficial moisture was removed by blotting with a dampened cloth. Fish length was measured with calipers from the tip of the closed mandible to the fork of the caudal fin. The time consumed in changing the fish from one tank to another, including the measurements, was 2 minutes or less. The duration of each of the main experiments to be reported here was 50 days (five periods, each of 10 days' duration).

Preliminary experiments were begun in the summer of 1950, when the investigation of two green sunfish was completed. All other fish were studied in the summer of 1951.

The water temperature ranged from 23°.5 to 25°.0 C. in 1950 (av. = 24°.5 C.), and from 24°.0 to 26°.5 C. in 1951 (av. = 25°.5 C.).

SAMPLES OF FISH AND MEAL WORMS

All analyses were performed on material dried to constant weight in a drying oven maintained at 95° C. Each sample was dried for at least 24 hours and usually longer.

Samples of fish were obtained by first drying the whole fish to constant weight, then grinding the whole body in a meat grinder until it was finely divided. A large aliquot portion was more finely ground in a mortar and stored in a drying oven at 70° C. for subsequent analysis.

Meal worms were dried to constant weight as described above. The whole meal-worm sample was ground in a mortar and stored in a drying oven.

FECES

The excrement which passed through the alimentary canal gradually accumulated on the bottom of the aquarium during each 10-day period. The aquarium water was filtered at the end of each period through a Büchner funnel under suction. Flakes of meal-worm integument made up most of the total weight recovered in this manner, but there was also a smaller portion of finely divided matter which was suspended in the water. As much material as possible was removed from the filter paper, after being dried and weighed to within 0.1 mg., by scraping the paper lightly with a knife. This material was used for analysis. The amounts of dry feces material were so small that it was necessary to lump several samples together for a nitrogen determination. Six such samples averaged 7.27 per cent nitrogen, with a standard deviation of ± 1.08 per cent. The standard procedure adopted for computing the amount of nitrogen in the feces was as follows: Mg. of nitrogen in feces = Dry weight of feces in mg. $\times$ 0.0727.

NITROGEN DETERMINATION

Determinations of nitrogen were made by following a standard procedure described by Niederl and Niederl (1942). A steam-distillation micro-kjeldahl apparatus of the Pregl-Parnas-Wagner design was used. All samples were run in duplicate, and appropriate blank values were obtained. The material to be investigated was first digested by the procedure recommended by Brüel *et al.* (1947).

ALIMENTATION

An essential part of the investigation was to learn whether the efficiency of food absorption varied among fish of different ages. The absorption efficiency of total nitrogen was calculated for each fish (Table 1) as follows:

$$\text{Absorption efficiency}^2 \text{ of total N in } \% = \frac{\text{Total N absorbed}}{\text{Total N consumed}} \times 100,$$

where

Total N absorbed = Total N consumed − total N in feces.

The longear sunfish were able to absorb between 91.3 and 95.5 per cent of the total nitrogen fed. A weighted average absorption of nitrogen by all longear sunfish was 94.0 per cent. Green sunfish varied in absorption efficiency from 89.2 to 94.4 per cent; the weighted average absorption of nitrogen was 92.3 per cent, slightly lower than longear sunfish. There was no demonstrable difference in absorption of total nitrogen between fish of different sizes in either species.

The foregoing analysis does not take into account the fact that a portion of the total nitrogen is incorporated into nonprotein material. Chitin is a nonprotein material containing nitrogen, which is found in meal worms. LaFon (1944) analyzed the integument of *T. molitor* larvae, and he found that the chitinous portion of the integument was 4.89 per cent (±1.1 per cent) of the dry weight of the animals. The chitin molecule is known to contain 6.9 per cent nitrogen (calculated from the molecular structure given by Richards, 1951, p. 16). The amount of nitrogen in the form of chitin which was contained in the food was calculated from this information (Table 2). Table 2 also gives an estimate of the efficiency of absorption of true protein on the basis that chitin is indigestible and makes up all the nonprotein nitrogen (NPN). There may be an additional quantity of NPN in the tissues of the larvae, but this quantity is unknown. It is presumed to be small. The nitrogen content of the chitin consumed may be subtracted from the total nitrogen consumed in order to obtain an estimate of the true protein consumed (col. 1 minus col. 3, Table 2). The feces of the fish were inspected closely, and a large proportion was found to be small pieces of larval integument. This was considered to be evidence that chitin is not digested by the fish. The same conclusion was reached by Mann (1948), who fed carp a mixture of Cladocera and copepods. The nitrogen content of the chitin consumed has been subtracted from the feces nitrogen for an estimate of the true protein egested (col. 5 minus col. 3, Table 2), since chitin is considered to be indigestible and therefore egested quantitatively.

The nitrogen considered as the egestion of true protein can arise from several sources: (1) breakdown of digestive enzymes, (2) alimentary tract tissue breakdown, (3) possible parasitic and symbiotic inhabitants of the alimentary tract which are eliminated, and (4) undigested protein. The portion of the feces composed of the first three items is not known, and they have been lumped together with undigested protein. This error is not considered to be large enough to influence the main conclusions, but all efficiencies of protein absorption will be underestimated. The mucous covering of the fish scales is occasionally sloughed off and is another possible source of nitrogen obtained by filtering the aquarium water. The quantity of mucus is very small, judging from an inspection of the aquaria, but the actual amount is not known. Mucus should be considered an excretory product and not a part of the feces, if it could be determined. A portion of the nitrogenous material of the feces may dissolve in the aquarium water. This dis-

[2] "Absorption efficiency" is equivalent to the term "coefficient of digestibility" which is commonly used in standard physiology and nutrition work. Coefficient of digestibility is objected to on the grounds that it is always expressed in per cent (not a coefficient) and that digestion does not necessarily result in absorption.

solved nitrogenous material should be regarded as egested material, but it could not be subjected to analysis.

The best estimate of the efficiency of protein absorption is found as follows (col. 7, Table 2):

$$\text{Efficiency of protein absorption in \%} = \frac{\text{Protein N absorbed}}{\text{Protein N consumed}} \times 100,$$

where

Protein N absorbed = Protein N consumed − protein N in feces.

A weighted average of the efficiencies of protein absorption by longear sunfish is 97.4 per cent; the same value for green sunfish is 95.7 per cent. The determination for LES 2 stands out as abnormally high, but efficiencies for the rest of the

TABLE 1

CONSUMPTION AND ABSORPTION OF TOTAL NITROGEN BY LONGEAR (LES) AND GREEN (GS) SUNFISHES FED MEAL WORMS IN 50-DAY AQUARIUM EXPERIMENTS

Fish	Beginning Dry Wt. of Fish* (G.)	Dry Wt. of Food Consumed (G.)	Total N in Food (Per Cent)	Total N Consumed (Mg.)	Total N in Feces (Mg.)	Total N Absorbed (Mg.)	Absorption Efficiency of Total N† (Per Cent)
LES 1.....	30.68	19.98	9.58	1,914	112	1,802	94.2
LES 2‡.....	17.19	16.88	9.58	1,615	72	1,543	95.5
LES 3a.....	7.92	4.68	9.58	449	39	410	91.3
LES 3.....	6.64	6.26	9.58	599	48	551	92.0
LES 4.....	2.56	5.80	9.58	556	39	517	93.0
GS 7.....	12.37	16.76	9.58	1,605	114	1,491	92.9
GS 1.....	3.54	6.36	10.15	646	59	587	90.9
GS 2.....	2.18	4.44	10.15	451	49	402	89.2
GS 5.....	1.59	7.07	9.58	677	38	639	94.4

* Based on water content of sample fish.
† See text for definition.
‡ Died after 30 days.

TABLE 2

ESTIMATION OF CONSUMPTION AND ABSORPTION OF PROTEIN NITROGEN BY LONGEAR AND GREEN SUNFISHES FED MEAL WORMS IN 50-DAY AQUARIUM EXPERIMENTS

Fish	N Consumed (Mg.) (1)	Chitin Consumed* (Mg.) (2)	N Consumed as Chitin† (Mg.) (3)	N Consumed as Protein (Mg.) (4)	N in Feces (Mg.) (5)	N in Feces as Protein (Mg.) (6)	Efficiency of Protein Absorption (Per Cent) (7)
LES 1....	1,914	977	67	1,847	112	45	97.6
LES 2....	1,615	824	57	1,558	72	15	99.0
LES 3a...	449	306	21	428	39	18	95.8
LES 3....	599	229	16	583	48	32	94.5
LES 4....	556	286	19	537	39	20	96.3
GS 7.....	1,605	821	57	1,548	114	57	96.3
GS 1.....	646	311	21	625	59	38	93.9
GS 2.....	451	217	15	436	49	34	92.2
GS 5.....	677	348	24	653	38	14	97.9

* Dry weight of food consumed in mg. × 0.0489.
† Chitin consumed in mg. × 0.069.

longear sunfish are in agreement. Green sunfish appear to be slightly less efficient in absorbing protein. This is due to the lower values for GS 1 and GS 2, which were studied in 1950 when the experiments were first begun. Equal care was taken to collect the feces of all fish, but the techniques in handling the aquarium water for filtration had not been perfected at this time, and a slight loss of material may have resulted. There is no difference in the efficiency of protein absorption by fish of different sizes.

TABLE 3

ESTIMATION OF PROTEIN NITROGEN USED FOR GROWTH BY LONGEAR AND GREEN SUNFISHES FED MEAL WORMS IN 50-DAY AQUARIUM EXPERIMENTS

Fish	Beginning Wet Wt. (G.)	H_2O Content* (Per Cent)	Beginning Dry Wt. (G.)	N of Dry Wt.* (Per Cent)	Beginning N Content (Mg.)	End Wet Wt. (G.)	H_2O Content (Per Cent)	End Dry Wt. (G.)	N of Dry Wt. (Per Cent)	End N Content (Mg.)	N Retained (Mg.)
LES 1	103.3	70.3	30.68	8.80	2,700	105.6	69.5	32.20	8.65	2,785	85
LES 2	57.9	70.3	17.19	9.22	1,585	70.2	66.5	23.55	8.28	1,950	365
LES 3a	28.2	71.9	7.92	9.26	734	31.5	71.6	8.95	9.62	860	126
LES 3	23.6	71.8	6.64	9.28	614	28.7	69.0	8.90	8.72	776	162
LES 4	9.1	72.0	2.56	9.26	237	15.3	68.0	4.90	8.36	409	172
GS 7	48.5	74.5	12.37	11.00	1,361	62.7	69.9	18.85	8.80	1,659	298
GS 1	18.2	80.5	3.54	10.61	376	22.3	73.7	5.87	9.58	562	186
GS 2	11.2	80.5	2.18	10.61	232	14.8	70.0	4.44	8.43	374	142
GS 5	7.1	77.4	1.59	10.62	169	17.3	69.9	5.20	8.12	422	253

* Based on sample fish analyses.

NITROGEN RETAINED BY THE FISH

A determination of the dry weight and percentage nitrogen content in the several sample fish at the beginning of the experiment allows an estimation of the amount of nitrogen which each experimental fish contained before feeding began. The result of this calculation is shown in Table 3. The nitrogen content of the experimental fish at the termination of feeding (50 days) also appears in the table. The amount of nitrogen retained by each fish is estimated by subtracting the quantity of nitrogen in each fish at the beginning of the experiment from the quantity of nitrogen at the end (last col. of Table 3). It is assumed that the NPN of the fish is the same at the beginning and end of the experiment, so that the retained nitrogen represents protein stored by the fish.

The nitrogen retained by the fish may be used (1) for growth (construction of new body tissue) and (2) for the nitrogen "reservoir." The nitrogen "reservoir" is not clearly understood by physiologists (Houssay, 1950; Best and Taylor, 1951), but it is thought to be that nitrogen which is circulating (or pooled) in the body fluids. A nitrogen reservoir has never been demonstrated directly for fish, but fasting experiments on trout by Morgulis (1919) hint that there might be a fair quantity of such nitrogen in the body. He found that there was a greater nitrogen excretion per unit time by fish at the beginning of a fast than at the end. The longest fasting experiment by Morgulis lasted 4 weeks, and the nitrogen excretion showed no evidence of leveling off. The 3-day fast which preceded our experiments probably reduced the nitrogen reservoir to some extent, but it is judged to be slight on the basis of Mor-

gulis' experiments. Therefore, the nitrogen retained by the fish in our experiments will be considered "growth" nitrogen.

A discussion of the gain in gross weight of the fish will be deferred until a later time. It may be mentioned that there was a considerable accumulation of fat in most fish during the experiments, and this influenced the weight relationships a great deal. The significance of the nitrogen used for growth will be discussed in the next section dealing with the nitrogen balance.

TABLE 4

PROTEIN NITROGEN BALANCE OF LONGEAR AND GREEN SUNFISHES FED MEAL WORMS IN 50-DAY AQUARIUM EXPERIMENTS

Nitrogen	LES 1	LES 2	LES 3a	LES 3	LES 4
Consumed (mg.)	1,847	1,558	428	583	537
In feces (mg.)	45	15	18	32	20
Absorbed (mg.)	1,802	1,543	410	551	517
For growth (mg.)	85	365	126	162	172
Residual (mg.)	1,717	1,178	284	389	345

Nitrogen	GS 7	GS 1	GS 2	GS 5
Consumed (mg.)	1,548	625	436	653
In feces (mg.)	57	38	34	14
Absorbed (mg.)	1,491	587	402	639
For growth (mg.)	298	186	142	253
Residual (mg.)	1,193	401	260	386

NITROGEN BALANCE AND UTILIZATION OF PROTEIN NITROGEN FOR GROWTH

It is now possible to construct a protein nitrogen balance from the best estimates available about the protein materials involved. Columns 4 and 6 in Table 2 and the last column of Table 3 have been retabulated in Table 4 to show the relationship between the protein (as represented by protein nitrogen) absorbed and that used (1) for growth and (2) for oxidative metabolism and tissue replacement. Neither the amount of protein required for the replacement of body tissue breakdown nor the protein used for oxidative metabolism has been determined. These two uses of protein have been lumped together as "residual N" in Table 4. The relationship between the protein nitrogen absorbed and the protein nitrogen used for growth by fish of different physiologic ages is the most significant feature of the nitrogen balance. The utilization of protein for growth has been determined as follows:

$$\text{Utilization of protein for growth in } \% = \frac{\text{Protein N used for growth}}{\text{Protein N absorbed}} \times 100.$$

The calculation has been used to construct Figure 1, which shows a close relationship between the beginning dry weight of the fish and the utilization of protein nitrogen for growth. As the body weight increases, the ability of the fish to use nitrogen for growth decreases. The utilization of protein by small longear sunfish of about 2 g. dry weight is 33 per

cent. Protein utilization decreases in a linear fashion to 5 per cent for the largest longear sunfish used (31 g. dry weight). Protein utilization by green sunfish appears to decline in a curvilinear fashion from about 38 per cent for fish of 1.7 g. dry weight to 20 per cent for the largest specimen (12.4 g. dry weight).

Four longear sunfish not heretofore mentioned are shown in Figure 1. These

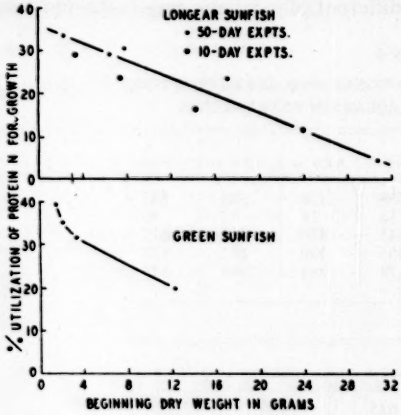

FIG. 1.—The relationship between beginning dry weight of longear and green sunfishes and the per cent utilization of protein for growth. The fish were fed meal worms in aquarium experiments which lasted 50 days (except those indicated).

fish were subjects of a 10-day experiment which was comparable to the 50-day experiments. The results of the short and long experiments are quite similar and confirm one another.

Although there are few fish represented in the figure, there is some justification for plotting the species separately. The difference in the relationship between body weight and protein utilization for the two species may be important. The curve bends upward for small-sized green sunfish, but it does not for longear sunfish. The difference in body weight between the smallest fish of the two species may account for these results; the smallest green sunfish weighed 0.97 g. (dry weight) less than the smallest longear sunfish. Possibly longear sunfish would have shown this characteristic if still smaller fish had been used. Some indication that fish of small size may be very efficient in converting available protein into body substance is shown by Ivlev's (1939*a*) work. He found that *Silurus glanis* larvae were about 67 per cent efficient in utilizing available energy for growth. The embryo of the chick compares favorably with the fish larvae, having an energetic efficiency of about 62 per cent (Needham, 1931).

No determinations of either protein utilization or energetic efficiency have been made for fish of maximum size up to this time. The largest longear sunfish used in our experiments was one of the largest ever collected near Bloomington, although hundreds of this species have been measured. It was probably VI years old, which is near maximum age in our geographic location. Even though such a large specimen was used, the protein utilization curve did not level off as it approached maximum size. The largest green sunfish was considerably smaller (probably age IV) than the longear sunfish and did not represent the maximum size of this species. Many larger green sunfish have been observed. One very large specimen was brought into the laboratory for experimentation, but it had a fungus infection and died 3 days later. The shape of the green sunfish curve suggests that fish of maximum size would have a low protein utilization. At the present time, it appears that a straight line would satisfactorily express the relationship between protein utilization and body weight within the range of 2 g. dry weight and maximum size.

The regression lines of the two species have different slopes. The longear sunfish shows a decrease in the utilization of protein of about 0.9 per cent per gram of

body weight increase. Judging from the inflection point of the green sunfish curve, there is a decrease in protein utilization of about 1.2 per cent per gram of body weight increase. This may be due to the fact that green sunfish *generally* do not grow so large as do longear sunfish. At least it might indicate that, of the fish which were studied, the green sunfish did not have so great a growth potential as did the longear sunfish.

TABLE 5

RELATION BETWEEN GAIN IN LENGTH AND PROTEIN RETAINED BY LONGEAR AND GREEN SUNFISHES FED MEAL WORMS IN 50-DAY AQUARIUM EXPERIMENTS

Fish	Beginning Length (Mm.)	Length at End (Mm.)	Length Gain (Mm.)	Increase (Per Cent)	Protein Retained* (G.)	Length Gain per G. Protein Retained (Mm.)	Protein Required for 1 Mm. Linear Growth (G.)
LES 1.....	154.0	156.0	2.0	1.3	0.53	3.77	0.265
LES 2.....	127.0	135.0	8.0	6.3	2.28	3.51	0.285
LES 3a....	107.0	112.0	5.0	4.7	0.79	6.33	0.158
LES 3.....	102.5	107.5	5.0	4.9	1.01	4.95	0.202
LES 4.....	75.0	89.0	14.0	18.7	1.07	13.08	0.076
GS 7.....	138.0	145.0	7.0	5.1	1.86	3.76	0.266
GS 1.....	99.0	106.0	7.0	7.0	1.16	6.03	0.151
GS 2.....	87.0	93.0	6.0	6.9	0.89	6.74	0.148
GS 5.....	77.0	95.0	18.0	23.4	1.58	11.39	0.088

*Protein N retained × 6.25.

The decreased utilization of protein for growth by fish of increasing size is a phenomenon which is probably associated with aging. Comparable examples for other animals are not known. Brody (1945) gives some values of energy utilization in growing domestic animals which indicate that the same phenomenon exists there. The utilization of protein for growth might be expected to decrease with age among animals with determinate growth. Animals with indeterminate growth have not been investigated in this respect, to the writer's knowledge. Fish are generally regarded as an animal group having indeterminate growth, because most fish grow throughout their lifetime. Our data indicate that the two species of sunfish which were studied have a rather definite growth limitation which would be at the point where no more (or at least an insignificant part) of the available protein could be used for growth. It is certainly true that different species have different growth *potentials*, and our results may in part explain this difference. It seems well established that the ability to use protein for growth is associated with aging in the green and longear sunfishes. The writer heartily agrees with Rasquin and Hafter (1951), who state: "The appearance of senility changes shows that teleosts conform to the common vertebrate pattern of aging despite a widespread misconception to the contrary."

RELATION BETWEEN PROTEIN RETAINED AND INCREASE IN BODY LENGTH

The increase in body length was determined by measuring the fish at the beginning and end of the experiment, as described under "Methods." Table 5 shows that the length increase of the longear sunfish varied from 18.7 per cent in the

small fish to 1.3 per cent in the large fish. The small green sunfish increased 23.4 per cent in length, while the largest gained only 5.1 per cent. Again a striking difference is apparent between old and young fish.

Growth in length is probably affected more directly by the retention of protein than by any other foodstuff, provided that the mineral materials necessary for skeletal development are present in suffi-

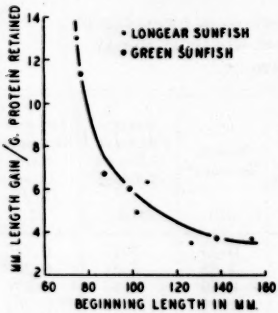

FIG. 2.—The relationship between length gain per gram of protein retained and the length of longear and green sunfishes at the beginning of 50-day aquarium experiments. The fish were fed meal worms.

cient quantity. Therefore, the length gain per gram of protein retained has been calculated for each fish (Table 5). This calculation brings out the great difference in the ability of fish of different sizes to use protein for linear growth. The length gain per gram of protein is extremely high for the smallest fish (11.39 mm. for green sunfish and 13.08 mm. for longear sunfish) in comparison to the largest fish (3.76 mm. for green sunfish and 3.77 mm. for longear sunfish). No striking difference between the species in this respect is apparent, and they have been plotted together in Figure 2. There is an asymptotic relationship between the variables of beginning length and length gain per gram of protein retained. The data were changed to logarithms and the regression coefficient was calculated as follows:

$$\log y = \log A + n \log x,$$

$$\log y = \log 20{,}140 + (-1.7486) \log x,$$

where

y = Length gain in mm. per g. protein,

A = Constant,

n = Regression coefficient,

x = Length of fish in mm. at beginning of experiment.

The regression coefficient (−1.7486) is 6.6 times its standard error (0.262), with seven degrees of freedom, indicating that the correlation is highly significant. Linear growth of these fish can, therefore, be interpreted as increasing by a constant fraction of the size already achieved. This reinforces the statement previously made to the effect that fish of different sizes are of different "physiologic ages," even though they may be of the same chronological age. Chronological age should not be eliminated altogether from consideration, because it undoubtedly plays a part in the ability of the organism to synthesize new body tissue. However, the concept of psysiologic age in fishes seems to be real and has not received attention up to this time.

The amount of protein necessary for a gain in length of 1 mm. (Table 5) is shown in Figure 3 as a function of the length of the fish at the beginning of the experiment. The linear relationship between these variables is puzzling, since protein growth would be a volume, or cubic, dimension, while length increase is a simple linear dimension. Perhaps this linearity would not hold true over the entire size range of fishes. From the data at hand, the following linear equation can be derived:

$$y = a + bx,$$

$$y = -0.0982 + 0.00261x,$$

where

y = G. protein required for 1 mm. growth in length,

a = Constant (y intercept when $x = 0$),

b = Regression coefficient,

x = Beginning length in mm.

The regression coefficient is 6.4 times its standard error (0.000407), with seven degrees of freedom. The correlation between the variables is statistically significant at the 1 per cent level. Using the relationship expressed above, it is possible to estimate the amount of protein necessary for any specified increment of growth in length.

Example.—The number of grams of protein required by a fish to grow from 70 to 140 mm. can be found by calculating the area of a trapezoid bounded by 70 mm., 140 mm., and the slope of the regression line. The area of the trapezoid is determined by multiplying the length of its base (increment of growth) by its average height. The average height of the trapezoid is the average of the values of y at 70 mm. (0.0845) and at 140 mm. (0.2672). The average height of the trapezoid becomes 0.1758 and the area: $70 \times 0.1758 = 12.41$ g. of protein required to grow an increment of 70 mm. between the limits of 70 mm. and 140 mm.

The relationship just described applies only to the data at hand, which are limited both in quantity and in quality. For example, the constant a in the regression formula is a negative number, which means that -0.0982 g. of protein is required for linear growth during the first stages of embryonic development. This, of course, is unrealistic. The manner in which the data were collected explains this discrepancy. The fish were killed a day after the final feeding. This did not allow them time to respond to the protein retained during the last phase of the experiment, and the determined values of length gain are low. This is also reflected in the calculation of protein required per unit of linear growth. Since the amount of protein necessary for linear growth is dependent upon the size of the fish at any instant of time, it is obvious that it should change during the course of 50-day experiments, where considerable linear growth was accomplished. The calculation of protein necessary for linear growth then represents only an average response of the fish—not a true instantaneous value. This average would tend to be lower than the true value. It would be difficult to determine the precise response of the fish to protein in terms of linear growth. Some of the difficulties are: (1) the time required for assimilated protein to be reflected in length increase would need to be determined; (2) much more precision in measuring length would be required; and (3) a more sensitive method for measuring protein retained by the fish would have to be devised. The last difficulty would prob-

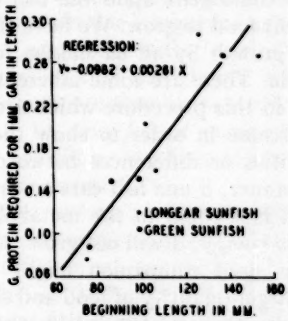

FIG. 3.—The relationship between the amount of protein required for a gain in length of 1 mm. and the length of longear and green sunfishes at the beginning of 50-day aquarium experiments. The fish were fed meal worms.

ably be the hardest to overcome. Perhaps the ideal conditions for these measurements could never be achieved, but they could be more closely approached than the experiments described here. However, our data do present a starting point and are valuable for estimation purposes.

DISCUSSION

The determination of protein utilization is contingent upon the fish eating sufficient food to grow. We have tried to insure growth by an *ad libitum* feeding schedule. There are some inherent difficulties in this procedure which are hard to overcome in order to show the true similarities or differences between fish. For instance, if one fish eats an amount of food just equal to the metabolic expense of energy, it will not grow. A comparably sized companion might eat a much larger quantity of food and show a rapid growth rate. Obviously, the first fish has not demonstrated its maximum growth capacity. The experimenter is faced with the problem of how much food the fish "should" eat. By manipulating the feeding schedule between a minimum and maximum amount, false conclusions could easily be reached. For this reason we have let the fish choose their own rate of feeding. The main differences between fish should then be due to individual response to the food in relation to what they can eat.

There may be an optimum amount of protein consumed, which would yield maximum growth. If this were known, it would partly solve the problem mentioned above. It is possible that the maximum amount of protein consumed might not lead to maximum retention for growth. The fish fed maximum rations by Ricker (1950) grew at a less rapid rate than fish fed slightly smaller amounts. Ricker's experiments were not detailed enough to establish the hypothesis of optimum protein intake, since the weight increase which he measured might have been complicated by fat accumulation. His experiments warrant repetition with regard to an analysis of the food and of the materials stored by the fish.

The reason why older fish should utilize protein less efficiently than young fish is difficult to explain. Probably this phenomenon is related to the question of why animals of determinate growth stop growing. It has been suggested that it is associated with "aging," but this is not a satisfactory answer. Several possible explanations come to mind which might be investigated. The size of the stomach and related structures of the alimentary canal might not increase in direct proportion to the size of the fish. Both surface area and volume of the digestive tract in relation to the weight of the fish would be important considerations in this respect. Possibly the digestive enzymes cannot supply the same amount of nutrient material per gram of body weight to fish of different sizes. Our data show that the efficiency of protein digestion is essentially alike in old and young fish. Lubich and Mann (1950) investigated trypsin in old and young herring, and they state that no differences between fish of different ages were found. They gave no data to support this statement, and it is not clear whether they determined the *quantity* or the *activity* of trypsin.

Metabolism (heat production) in our fishes increased with increasing body size (Gerking, MS), as is true of all animals. Possibly the decreased protein utilization is associated with the change in metabolism or in some of the bodily processes which control metabolism. The thyroid hormone is commonly associated with metabolism and may have a direct influence on the conversion of nutrient protein to body substance. Fish may be good

material for investigation of this sort, because their physiology is less complicated by puberty, periods of accelerated growth, metamorphosis, or rapidly fluctuating reproductive cycles that are found in the higher vertebrates.

SUMMARY AND CONCLUSIONS

1. The utilization of protein for growth by fish of different ages was studied. Longear sunfish (*Lepomis megalotis megalotis*) and green sunfish (*L. cyanellus*) were kept in aquaria and fed a weighed amount of meal worms (*Tenebrio molitor* larvae) each day for 50 days. Nitrogen retention was measured by the difference in nitrogen content between the experimental fish at the end of the experiment and a sample fish of the same size at the beginning of the experiment. Feces nitrogen was measured by filtering the aquarium water every 10 days and analyzing the residue. The absorption of protein was estimated by subtracting the protein nitrogen in the feces from the protein nitrogen of the food consumed. The utilization of protein for growth was measured by relating the protein nitrogen retained to the protein nitrogen absorbed.

2. There was no difference in the ability of fish of different ages to absorb protein. Efficiencies of protein absorption averaged 97.4 per cent for longear sunfish and 95.7 per cent for green sunfish.

3. Young longear sunfish (10 g. live weight) utilized about 33 per cent of the protein they consumed for growth. Green sunfish of the same size had a comparable utilization. The oldest longear sunfish (105 g.) utilized 5 per cent of the available protein for growth, while the largest green sunfish (55 g.) utilized 20 per cent of the protein absorbed into the body for growth. The largest green sunfish did not approach maximum size for the species, while the largest longear sunfish did.

4. The fact that fish are able to utilize less of the available protein for growth as they increase in size is regarded as a phenomenon associated with "aging." It is also suggested that fish growth is much more determinate than it is usually considered to be, since fish of maximum age are able to utilize very little of available protein for growth.

5. There was an asymptotic relationship between length gain per gram of protein used for growth and the length of the fish at the beginning of the experiment. The young fish increased about 12 mm. in length for each gram of protein used for growth, while old fish increased about 3.5 mm. for each gram of protein stored.

6. The amount of protein required for 1 mm. of linear growth was shown to be directly related to the length of the fish at the beginning of the experiment. These data are subject to certain criticisms, which are discussed. Nevertheless, they are useful for estimating the amount of protein required to grow a specified increment in length.

7. The shape of the protein utilization curve was discussed, along with the possible factors influencing it.

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HORMONAL CONTROL OF METABOLISM IN CRUSTACEANS

IV. RELATION OF TISSUE COMPOSITION OF HEMIGRAPSUS NUDUS TO INTERMOLT CYCLE AND SINUS GLAND[1]

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THERE is now abundant evidence that the eyestalks of crustaceans contain endocrine organs which secrete a molt-inhibiting hormone (Brown and Cunningham, 1939; Scudamore, 1947; Edwards, 1950; Passano, 1951). This hormone is present in the sinus gland, but recent evidence suggests that it is elaborated by the *x*-organ and merely stored in the sinus gland (Bliss, 1951; Havel and Kleinholz, 1951; Passano, 1951). The movements of calcium into and out of the various tissues of the crustacean body constitute an important aspect of the molt cycle, and a number of studies have examined the effects of extirpation of sinus glands or eyestalks on calcium metabolism, especially the effects on deposition of calcium in the gas-

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troliths of crayfish (Brown, 1942; Kyer, 1942; Scudamore, 1942) and on the calcium content of crayfish blood (Havel and Kleinholz, 1951). Removal of the eyestalks results in rapid formation of gastroliths, and a decrease, followed by a marked increase, in blood calcium. Sinus gland removal has similar effects on gastrolith formation, but the increase in blood calcium is not evident. It has been proposed, without conclusive evidence, that the various metabolic actions of the eyestalk hormone (or hormones) may be referred primarily to the effect on calcium metabolism (Edwards, 1950).

None of the studies noted above has taken into account the fundamental work of Drach (1939), who showed that the intermolt cycle in crustaceans constitutes a regular succession of stages and that calcium metabolism varies in the different stages. Drach, on the other hand, was not aware of the significance of the sinus gland hormone in the regulation of the molt cycle. The question therefore arises, To what extent are the variations in metabolism of calcium during the intermolt cycle influenced by the eyestalk hormone? The present investigation is aimed primarily at this question. We have determined the calcium content of the digestive gland and the carapace of the shore crab *Hemigrapsus nudus* in each of the early stages of the intermolt cycle, during which the integument is being completed. We have also examined the effect of sinus gland extirpation on the calcium content of the tissues during certain of these stages. In the course of these observations, information was also obtained concerning the content of water, ash, and organic matter in the tissues.

MATERIALS AND METHODS

Specimens of *H. nudus* were collected in the vicinity of Newport, Oregon, and Charleston, Oregon. They were transported to the laboratory at Charleston or Eugene, their stage in the intermolt cycle was determined, and they were dissected as soon as possible after collection. When it was necessary, in the extirpation experiments, to maintain the animals in the laboratory at Eugene, they were kept in individual pint jars in shallow sea water; they were fed fish scraps each evening, and the water was changed each morning. It was possible to maintain the animals in good condition in this way for several weeks, but none of the experiments reported here involved maintenance for more than a week after collection.

The criteria for determining the stage in the intermolt cycle were based on those of Drach (1939) and Hiatt (1948). The criteria actually used are given in Table 1. Animals were divided into groups on the basis of these criteria, and the animals in each of the groups were then re-examined; the reliability of the method proved to be high. The animals were then dissected; the carapace was removed, adhering tissue was trimmed off, the carapace was blotted dry with paper and weighed on a torsion balance. The digestive gland was removed, blotted, and weighed on the same balance. The tissues were then dried in a vacuum oven for 4 hours at 110° C. and weighed again. The difference between the wet and dry weight is reported in the results as per cent water loss.

The dried tissues were then transferred to crucibles and ashed in a muffle furnace at 500° C. for 3 hours; the weight of ash was determined and is reported as per cent ash. Ten-milligram samples of ash were transferred to 15-ml. centrifuge tubes and were dissolved in a minimal amount of 6 N HCl; the Kramer-Tisdall method for calcium, involving precipitation as oxalate and permanganate titra-

tion of the oxalate (Clark and Collip, 1925), was used, with slight modification to minimize precipitation of magnesium and to allow for the relatively large amounts of calcium present.[2] The results are reported as per cent of calcium in the tissue. The per cent of organic matter was estimated as 100 minus the sum of per cent water and per cent ash. All the percentages, except per cent calcium in the ash, are referred to the wet weight of the tissue. An estimate of the sources of variation in the chemical determinations is given in Table 2.

The results were analyzed statistically by the method of analysis of variances (Dixon and Massey, 1951). A two-way classification, by operation and intermolt cycle stage, was utilized in the analysis of the effects of sinus gland removal. Because of accidental loss of animals and unequal numbers of sexes, the numbers in the various classes were unequal, and it was necessary to use an analysis based on proportional numbers. This necessitated the removal of data from some groups to make the numbers proportional. An analysis of the groups from which the largest number of items had been discarded was also carried through on a basis of unequal numbers, without discarding any items. The results of the two analyses were not significantly different.[3]

TABLE 1*

CHARACTERISTICS OF THE STAGES OF THE INTERMOLT CYCLE OF *Hemigrapsus nudus*

Stage	Carapace						Thoracic Sternites	Carpus and Propodus of Chelipeds	Merus and Carpus of Walking Legs	General Characteristics
	Protogastric Area	Meso- and Urogastric	Anterior Branchial Area	Posterior Branchial Area	Branchiostegites	Cardiac Area				
A_1	++	++	++	++	++	++	++	++	++	Period of absorption of water, immediately after molt, and of calcification
A_2	+	++	+ to ++	++	++	++	++	+	++	
B_1	−	++	+	++	++	+ to ++	+	−	++	Water absorption and calcification complete; period of hardening of integument
B_2	−	+	− to +	++	++	+	+	−	++	
C_1	−	+	+	− to +	+	+	− to +	−	+	Period of completion of integument, feeding, and tissue growth
C_2	−	−	−	−	−	+	−	−	+	
C_3	−	−	−	−	−	−	−	−	−	
C_4	.....	.....	.....	.....	.....	.....	.....	.....	.....	Membranous layer present
D	.....	.....	.....	.....	.....	.....	.....	.....	.....	New integument forming; period of preparation for molt

* ++ = integument completely soft; + = integument firm but easily compressed; − = integument rigid.

[2] We are indebted to Dr. D. F. Swinehart, of the Department of Chemistry, for helpful suggestions regarding the analytical method.

[3] We are indebted to Dr. W. J. Dixon and Mr. C. K. Tsao, of the Department of Mathematics,

The sinus gland was removed by boring a circular hole in the eyestalk integument directly over the region of the sinus gland with a hand dental drill. The overlying soft tissues were carefully pushed aside with a dissecting needle, and the sinus gland aspirated into a micropipette. No attempt was made to close or cauterize the wound, but the postoperative mortality was very low.

TABLE 2*

ESTIMATE OF SOURCES OF VARIATION (σ^2) FOR THE ESTIMATION OF CALCIUM

Sources of Variation	σ^2	
	Carapace	Digestive Gland
Dry weights	Negligible	0.043
Ash weights	0.0065	0.17
Calcium determinations	4.21	0.71
Variation in animals	2.98	14.73
Total	7.20	15.65

* The values for the mean squares (σ^2) for dry weight, ash weight, and calcium determination are based on variation between replicate determinations. The total mean square is taken from the variance within subclasses of the per cent calcium in the ash of the respective tissues. The variation in animals is the total less the mean squares for dry weight, ash weight, and calcium determination.

RESULTS

The results of the analyses of the carapace of normal animals in the first seven stages of the intermolt cycle are presented in Table 3. The first stages exhibit a progressive decrease in water content and increase in ash and organic matter. The increase in ash (and calcium) occurs primarily in the early stages (A_1–B_1), while the increase in organic matter occurs largely in the transition from B_2 to C_1. The ash content in the first postmolt stage (A_1) is already quite high; this is in contrast to the results of Schwabe *et al.* (1952) with the spiny lobster *Panulirus japonicus*, where the ash content of lobsters in stage B was below 3 per cent. It is evident also that the deposition of organic matter is not the laying-down of an organic matrix for mineral deposition; this occurs in the D stages, before the molt. The deposition observed here is concerned in the completion of the integument after mineralization is substantially complete.

The course of events in the digestive gland (Table 3) is quite different, as one would expect. There is a sharp decline in total calcium content between the A_1 and A_2 stages, when mineralization of the carapace is occurring. With the virtual completion of mineralization, in stage B, the calcium content of the gland rises, to undergo a second sharp decline in stage C_1. This second sharp decline is in part a consequence of a decrease in total ash, but it also results from a marked decrease in the percentage of calcium in the ash. Evidently, the beginning of stage C_1 is marked by a replacement of calcium by other inorganic ions in the digestive gland. The water content of the digestive gland rises to a maximum in stage B_1 and then declines, reaching about the same level in C_3 that it had in A_1. The organic matter, moderately high in the A stages, reaches a minimum in stage B and rises to a maximum well above its initial values in C_3. The C stages are marked by a deposition of organic matter in both carapace and digestive gland; this is to be expected, since the C stages are the stages of feeding and active tissue growth (Drach, 1939). The minimum in stage B may represent utilization of organic matter from the digestive gland for formation of integument, at a time when feeding has not yet begun.

In view of the facts (1) that the C stages are the longest in the intermolt cycle (Drach, 1939; Hiatt, 1948) and (2) that the transition from B_2 to the C_1

for valuable suggestions and assistance with the statistical analysis.

stage is marked by the most striking changes in tissue composition, we decided to perform the operation of sinus gland removal on animals in the C stages, anticipating that the effects of the operation would be most marked in these stages. The animals were maintained in the laboratory for 7 days following the operation, and then were analyzed together with normal controls. The results are given separately for males and females because in some cases a difference in the effect of operation between the sexes was evident.

TABLE 3

CHANGES IN COMPOSITION OF CARAPACE AND DIGESTIVE GLAND OF *Hemigrapsus nudus* DURING THE EARLY STAGES OF THE INTERMOLT CYCLE*

VALUE (PER CENT)	STAGE						
	A_1	A_2	B_1	B_2	C_1	C_2	C_3
Carapace:							
Water	52.8	47.5	39.9	33.2	30.2	27.9	26.7
Ash	35.6	41.2	43.5	43.2	45.3	44.9	44.7
Calcium in ash	63.0	62.4	64.5	62.9	64.0	62.4	59.0
Calcium in tissue	22.4	26.8	28.4	27.7	28.8	28.4	26.6
Organic matter	11.7	11.4	16.6	13.6	24.6	27.2	29.6
Digestive gland:							
Water	66.7	69.7	74.2	73.0	72.6	67.3	65.6
Ash	17.1	10.7	14.6	12.4	8.95	8.25	5.38
Calcium in ash	23.0	23.8	21.1	19.5	6.88	12.4	8.55
Calcium in tissue	4.81	1.42	3.00	2.63	0.76	0.86	0.55
Organic matter	16.2	19.6	11.2	14.6	18.5	24.5	29.0

* For statistical analysis see Table 4.

TABLE 4

STATISTICAL ANALYSIS OF DATA OF TABLE 3 (ANALYSIS OF VARIANCE)

VALUE	SOURCE OF VARIATION			
	Within Subclasses		Stage Means	
	Degrees of Freedom	Mean Square	Degrees of Freedom	Mean Square*
Carapace:				
Water	76	24.64	6	1,145.88†
Ash	39	2.50	6	56.56†
Calcium in ash	55	7.20	6	20.67‡
Calcium in tissue	25	0.86	6	20.37†
Digestive gland:				
Water	73	11.01	6	186.74†
Ash	42	19.13	6	92.63†
Calcium in ash	59	15.65	6	428.04†
Calcium in tissue	33	0.78	6	11.97†

* In this column, † indicates highly significant value ($F < 1$ per cent), ‡ indicates significant value ($F < 5$ per cent).

The results for the carapace are not presented in detail; no significant changes in water content, per cent ash, or per cent calcium were observed as a result of the operation. It is of interest, however, that the organic matter content of the carapace of operated animals was in each stage distinctly higher than that of normal animals, ranging from 28.5 per cent in stage C_1 to 32.9 per cent in stage C_3, as compared with normal values of 24.6 and 29.6 per cent. This is in agreement with the conclusion of Schwabe *et al.* (1952) that the eyestalk hormone prevents the deposition of organic matter in the carapace of the spiny lobster. In the digestive gland, removal of the sinus gland produced significant changes in water content, per cent ash, and per cent calcium in the ash. Removal of the sinus gland from males in stage C_1 caused a drop in water content to levels characteristic of C_2 and C_3; the effect was less marked in males in C_2 and C_3. In females, distinct decreases in water content were observed in all three stages. The decreases in water content are related to an increase in organic matter content observed in all three stages. Evidently, removal of the sinus gland accelerates the normal trend of replacement of water by organic matter.

The effect of sinus gland extirpation on digestive gland ash was remarkable, in that it resulted in an increase in per cent ash in stage C_1 and a decrease in stage C_2. This appears as a significant interaction between treatment means and stage means in Table 6; a similar interaction is apparent in Table 5 for males, but it fails to be statistically significant because of the high individual variability in the males. Sinus gland extirpation resulted in an increase in the per cent calcium in the ash in all three stages, but especially in stage C_1; the levels attained are those characteristic of earlier stages in the intermolt cycle (A_1–B_2). The combined effect of the changes in ash content and composition is to produce a marked increase in calcium content of the digestive gland in stage C_1, with little change in stages C_2 and C_3.

All the changes resulting from sinus gland removal appear to be more pronounced in stage C_1 than in the later stages; in the case of mineral content, the increase is apparent only in this stage. This suggests that the endocrine activity of the gland is at a maximum in this stage and decreases progressively as the next molt approaches.

DISCUSSION

Drach (1939) has shown that the intermolt cycle of crabs can be divided into four periods, as follows: (A) period of absorption of water and increase in weight, immediately following the molt; (B) period of hardening of the integument; (C) period of completion of integument and tissue growth; (D) period of preparation for molt. Each of these periods is further subdivided into stages on the basis of changes in the integument. In *H. nudus*, period A is characterized also by calcification of the integument, which is virtually completed by the beginning of period B, although the carapace is not yet fully hardened. Hardening of the crustacean integument depends only partly on calcification and also involves quinone tanning, as in insects (Dennell, 1947). In *Panulirus argus*, calcium is apparently deposited after exuviation in the layers formed before exuviation and is further deposited in the postexuvial layers as they are formed in later periods (Travis, 1951). This is in agreement with the observations of Schwabe *et al.* (1952) that the integument of *P. japonicus* contains very little ash immediately after the molt. However, *H. nudus* and probably many other hard-shelled crabs ex-

hibit a high mineral content in the integument early in period A, and mineralization is completed rapidly after molting. The sinus gland has no effect on the integumentary minerals in period C; it does, however, appear to retard the deposition of organic matter in the integument.

The successive stages of periods A, B, and C are marked by a gradual replacement of the water absorbed after molting in stage A_1 by organic matter; this process is interrupted in period B by the utilization of organic matter from the digestive gland, probably to supply material for formation of integument. The replacement is apparently retarded by the sinus gland, since extirpation of the gland results in an accelerated deposition of organic matter. The active principle of the gland therefore acts as an inhibitor of growth and assimilation. The onset of the stage of tissue growth (C_1) is also marked by a sudden decrease in the calcium con-

TABLE 5

EFFECT OF SINUS GLAND REMOVAL ON COMPOSITION OF DIGESTIVE GLAND OF *Hemigrapsus nudus* DURING STAGE C OF INTERMOLT CYCLE*

VALUE (PER CENT)	SEX	NORMAL			OPERATED		
		C_1	C_2	C_3	C_1	C_2	C_3
Water	M	72.5	64.1	62.6	63.1	61.9	61.7
	F	76.5	69.5	66.2	69.8	64.5	61.7
Ash	M	7.70	10.2	6.60	13.2	6.77	5.35
	F	5.40	7.38	4.93	14.8	3.95	5.68
Calcium in ash	M	9.08	17.4	12.7	23.6	19.7	20.8
	F	4.40	8.40	9.12	20.8	17.4	19.2
Organic matter	M and F	18.5	24.5	29.0	20.5	30.8	31.6

* For statistical analysis see Table 6.

TABLE 6

STATISTICAL ANALYSIS OF DATA OF TABLE 5 (ANALYSIS OF VARIANCE)

VALUE	SEX	SOURCE OF VARIATION							
		Within Subclasses		Interaction		Stage Means		Treatment Means	
		Degrees of Freedom	Mean Square	Degrees of Freedom	Mean Square*	Degrees of Freedom	Mean Square*	Degrees of Freedom	Mean Square*
Water	M	18	25.41	2	44.44	2	81.55	1	183.63‡
	F	14	10.44	2	1.02	2	77.15†	1	121.03†
Ash	M	16	17.06	2	47.67	2	29.76	1	5.65
	F	12	5.53	2	34.54‡	2	19.17	1	0.16
Calcium in ash	M	16	23.62	2	84.47	2	10.91	1	438.09†
	F	12	11.85	2	12.06	2	4.03	1	478.23†

* For values of entries in this column see Table 4.

tent of the ash of the digestive gland to about half its value in periods A and B. We may suppose that this change is a result of the action of the sinus gland principle, since it is as suddenly reversed by extirpation of the sinus gland. We may also infer that the sinus gland principle comes into action at the beginning of period C; this is consistent with the fact that period C is by far the longest in the intermolt cycle (Drach, 1939). It would be premature to hazard any guess as to the basic mechanism of action of the sinus gland principle at the present time.

It is obviously necessary, to substantiate the hypothesis proposed in the preceding paragraph, to have information as to the effects of sinus gland removal in periods A, B, and D of the intermolt cycle. Experiments to determine this are now in progress, as are studies in the nature of the organic matter deposited during period C. It would also be of interest to determine what cations replace calcium in the digestive gland in period C.

One object of the present investigation, at its outset, was to develop a method for assay of the sinus gland principle. This was not accomplished, but it appears reasonable that a measure of the per cent of calcium in the ash of the digestive gland would afford the most reliable basis for such an assay.

SUMMARY

1. Measurements of the water and ash content and of the calcium content of the ash and calculations of the calcium and organic matter content of the carapace and digestive gland of the shore crab *H. nudus* are presented for the first seven stages of the intermolt cycle.

2. Mineralization of the carapace occurs rapidly after molting and is complete long before the structure of the carapace is complete; deposition of organic matter in the carapace occurs for some time after mineralization is complete.

3. The relative content of organic matter in the digestive gland falls soon after molting, and then rises, as feeding and tissue growth begin.

4. Calcium is mobilized to some extent from the digestive gland during the period of mineralization of the integument; with the onset of the period of feeding and active growth, there is a sharp decrease in the calcium content of the gland, resulting from a decrease in per cent ash and a decrease in the proportion of calcium in the ash.

5. Sinus gland removal results in a marked increase in the ash content in stage C_1, and a marked increase in the calcium content of the ash in stages C_1, C_2, and C_3; the ash content is decreased in stage C_2. The effect of sinus gland removal is therefore to increase the calcium content of the digestive gland only in stage C_1.

6. Sinus gland removal also results in increased deposition of organic matter in the digestive gland and carapace.

7. It is proposed that the action of the sinus gland principle is normally initiated at the beginning of stage C_1 and that it acts to inhibit deposition of calcium in the digestive gland and to inhibit tissue growth and deposition of organic matter.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

THE STUDY OF INSTINCT[1] BY N. TINBERGEN

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SINCE the middle 1930's, Konrad Lorenz, N. Tinbergen, and their pupils have been making remarkable progress in analyzing the physiological basis of instinct. Their ethological theory has won wide acceptance in Europe, but across the Atlantic its influence is still surprisingly small, partly because most of the literature is in German but mainly because instinct is currently unpopular in American psychology.

Dr. Tinbergen is well known in the United States for his efforts to publicize ethology. His present book grew out of a series of lectures delivered in New York during 1947. Describing his aims, the author says: "By presenting a review of this work in the English language I hope to contribute to international co-operation in the science of animal behavior. Second, this book is an attempt at an organization of the ethological problems into a coherent whole" (p. v).

The first five chapters are concerned with the underlying external and internal factors and are followed by brief but original treatments of the development, adaptiveness, and evolution of behavior. The style of writing is refreshingly clear; numerous examples and neat sketches enliven the discussion. There is an extensive Bibliography, an Author Index, and a Subject Index limited to listing the animals mentioned in the text.

After a survey of sensory capacities, Tinbergen shows by ingenious experiments with models that animals react instinctively to a very small part of the stimuli they are capable of perceiving. Since only these sign stimuli are usually effective in releasing a reaction, there must be an inborn neurosensory mechanism (the "Innate Releasing Mechanism") which is specialized to respond to a particular stimulus combination. The IRM integrates various afferent stimuli "in a purely quantitative way. . . . In other words, either before they reach the motor center or in the motor center itself, the effects of the external stimuli are brought together and added" (p. 81). However, internal factors (humoral and neural) influence thresholds of response, not through the IRM, but by directly affecting the central nervous system. They may induce a central accumulation of "action-specific energy" (Lorenz), which can at times lead to explosions of "vacuum activity."

Instinctive acts are combinations of rigidly fixed motor patterns (*Erbkoordinations*) and more variable components, which Lorenz called "taxes." Tinbergen defines "taxis" as "the integration of reflex-like reactions, responsible for the orientation," and the fixed pattern as "an integration of muscle contractions controlled from within" (p. 88). D. L. Gunn has objected to this use of "taxis" on the grounds that it has acquired greater utility in the limited field of "simple" oriented movements. Still the redefinition seems soundly profitable. It has already led Tinbergen to conclude that the dorsal light reaction is a form of tropotaxis and that the light compass reaction, telotaxis, menotaxis, and pharotaxis are essentially similar phenomena.

The author's "center theory" of instinct assumes that Erbkoordinations are controlled by subinstinct centers of a higher order or level, and these in turn are co-ordinated by still higher level centers. The topmost is "loaded" by internal neural and hormonal stimuli, but each of the lower centers is held to be blocked until key external stimuli, by affecting the IRM, remove the block of one of the subinstinct centers, permitting it to load the centers of the Erbkoordinations which it controls. Since the latter are also blocked, further key stimuli are needed to activate each of them. Finally, the released Erb-

[1] Oxford: Oxford University Press, 1951. Pp. 228. $7.00.

koordinations (with their taxes) become "consummatory acts" (after W. Craig) in the double sense of end-reactions and energy-dissipating responses.

At each level, there is "appetitive" behavior —the variable, highly purposive activities that accompany instinctive acts. When the external stimuli needed to deblock a lower center are lacking, impulses flow to the pathways of appetitive behavior, resulting in a form of searching, with the goal of finding the appropriate releasers. G. P. Baerends has shown (in the wasp, *Ammophila*) that deblocking of a subinstinct center leads again to more limited appetitive behavior until the Erbkoordinations are activated. Thus there is a hierarchy not only of drives but of appetitive behavior as well.

Tinbergen defines instinct as a "hierarchically organized mechanism which is susceptible to certain priming, releasing and directing impulses of internal as well as external origin, and which responds to these impulses by coordinated movements that contribute to the maintenance of the individual and the species" (p. 112). Emphasizing the tentativeness of the picture, he lists with some hesitation the major instincts of reproduction, escape, eating, sleep, and comfort (care of the surface of the body) but rules out a separate social instinct, assuming that social behavior occurs only as part of other instincts.

The development of behavior (chap. vi) is considered broadly to include growth, maturation, and learning; the latter is classified for the purpose as "a phenomenon of ontogenetic growth of behavior superimposed on the innate patterns and their mechanisms" (p. 128). The discussion of growth is based almost entirely on the amphibian work of Coghill, Carmichael, and Weiss, which fits the instinct theory better than do most of the omitted avian and mammalian data. Some exceedingly interesting evidence is presented that there are innate predispositions to learn certain things and not others, at definite places in the instinctive patterns and at special times in development.

By "adaptiveness" (chap. vii) is meant contributing to the maintenance of individual or species. Innate behavior, in combination with the structural mechanism which it serves, often enables the animal to do "the right thing at the right moment." Numerous cases are cited of adaptive behavior in feeding, escape, and postural attitudes relating to protective concealment. The second half of this chapter discusses thoroughly the highly adaptive social releasers.

The evolution of behavior (chap. viii) is far more difficult to study than that of morphological changes. Almost all the evidence must come from homology, which Tinbergen seems to be using as *similarity of origin* in related species. He suggests that, "once morphology has succeeded in giving us a picture of the phylogenetic relationships within a group, we may take this picture for granted, and compare the behavior of the species concerned. Or, starting with behavior we might arrive at a tentative picture of the evolution of a certain type of behavior and check this picture with the conclusions of morphological study" (p. 193). Morphologists also use rather indefinite criteria for homology, the specialist tending to rely on his insight into a taxonomic group. It would seem uncritical for an observer to depend solely on his impressions, but the fixed patterns are very constant and are easily compared when a large enough series of related forms is studied.

Displacement activities have proved to be very useful tools in the study of homologies. They result from a surplus of motivation, chiefly during conflicts of drives within the animal, with a consequent "jumping-over" to the pathways of still another drive. For instance, when the surplus is from aggressive or sexual drive, the animal may show "irrelevant" movements of feeding, preening, or even sleeping! Comparison of displacements in related species sometimes reveals striking evolutionary sequences, with the addition of new functions as social releasers. The complex social displays of many animals have developed in large part from such "derived" activities (N. Tinbergen, *Quart. Rev. Biol.*, **27**:1–32, 1952).

The tracing of homologies is the first step in studying the genetics of innate behavior and its speciation. Behavior plays a prominent role in isolating mechanisms, and in both inter- and intraspecific selection. Only fragmentary evidence is available in this intriguing area.

The closing section applies the instinct concept to man. This is made difficult by two general defects in ethology: mammals have been almost neglected, and learning has not been studied systematically to separate its effects from instinct. It can hardly be questioned that instinctive acts, innate releasing mechanisms, and displacement activities exist in man, but at present we cannot assess their importance as compared with learning.

In its current stage, ethology resembles a

patchwork quilt, pieced together with bits of stickleback, wasp, cichlid, duck, herring gull, etc., the whole seeming "to cover the reality better than any theory thus far advanced." Until more facts are available, some of the hypotheses may be viewed with skepticism, but they have stimulated much productive research and will continue to do so in the future. Tinbergen's fine book is an important synthesis and a valuable introduction to this actively moving field.

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